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Trophic transfer of copper decreases the condition index in *Crassostrea gigas* spat in concomitance with a change in the microalgal fatty acid profile and enhanced oyster energy demand

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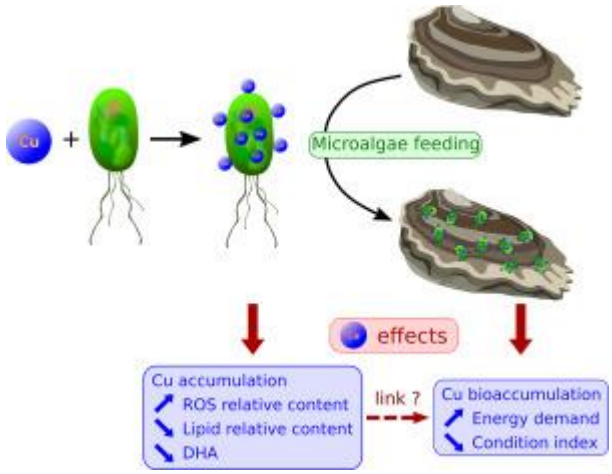
Abstract :

Due to new usages and sources, copper (Cu) concentrations are increasing in the Arcachon Basin, an important shellfish production area in France. In the present paper, the trophic transfer of Cu was studied between a microalga, Tetraselmis suecica, and Crassostrea gigas (Pacific oyster) spat. An experimental approach was developed to assess Cu exposure, transfer and toxicity on both phytoplankton and spat. Exposure of microalgal cultures to Cu for 7–8 days (3.1 ± 0.1, 15.7 ± 0.2 and 50.4 ± 1.0 μg Cu·L−1 for the control, Cu15 and Cu50 conditions, respectively) led to concentrations in microalgae (28.3 ± 0.9 and 110.7 ± 11.9 mg Cu·kg dry weight−1 for Cu15 and Cu50, respectively) close to those measured in the field. Despite Cu accumulation, the physiology of the microalgae remained poorly affected. Exposed cultures could only be discriminated from controls by a higher relative content in intracellular reactive oxygen species, and a lower relative content in lipids together with a reduced metabolic activity. By contrast, the fatty acid profile of microalgae was modified, with a particularly relevant lower content of the essential polyunsaturated fatty acid 22:6n-3 (docosahexaenoic acid [DHA]). Following 21 days of spat feeding with Cu15 and Cu50 microalgal cultures, trophic transfer of Cu was observed with a high initial Cu concentration in spat tissues. No effect was observed on oxidative stress endpoints. Cu exposure was responsible for a decrease in the spat condition index, an outcome that could be related to an insufficient DHA supply and extra energy demand as suggested by the overexpression of genes involved in energy metabolism, ATP synthesis and glycogen catabolism.

Graphical abstract



Highlights

► Contaminated *T. suecica* cultures reached an environmentally relevant load of Cu. ► Cu impacted the ROS content in algae, alongside their fatty acid profile. ► Trophic transfer of Cu led to spat contamination close to what occurs in the field. ► Spat fed with Cu-exposed algal cultures had a lower condition index. ► Insufficient supply of PUFA 22:6n-3 and elevated energy demand may explain this effect.

Keywords : Cu, Tetraselmis suecica, oyster, trophic exposure, toxicity, lipid composition

1. Introduction

Coastal waters are contaminated by a high diversity of inorganic and organic chemical pollutants from various origins. Aquaculture areas, including oyster and mussel production basins, are particularly susceptible to chemical pollution due to their location close to the coast. Inputs of pollutants such as herbicides have already been detected in the Marennes Oléron Basin (REMPAR, 2018; Tapie and Budzinski, 2018), one of the most important sites in France for collecting oyster seeds by remote setting. Herbicides reach coastal waters from the exoreic Charente catchment area, which is characterised by important agricultural activity (Munaron et al., 2006). Further south, the Arcachon Basin is also an important site of oyster spat production in France; it is known to be contaminated by various pollutants such as polycyclic aromatic hydrocarbons (PAHs), polychlorobiphenyls (PCBs), tributyltin (TBT) (Claisse and Alzieu, 1993; Devier et al., 2005) and copper (Cu) (Trut et al., 2013). Moreover, a long list of chemicals (e.g. trace metal elements, radionuclides, benzo[a]pyrene, PCBs, dioxins and furans, PAHs, TBT, hexachlorobenzene [HCB], dieldrin, lindane, triazines and polybrominated diphenyl ethers) have been detected in shellfish along the French coast, attesting to their exposure to a high diversity of chemical pollutants (Guéguen et al., 2011). Chemical pollution has thus been considered one of the many factors responsible for high mortality events and reproduction impairment affecting some bivalve species that have been observed for several years (Samain and MacCombie, 2008). Among them, the Pacific oyster, *Crassostrea gigas*, is one of the foremost aquaculture resources around the world (FAO, 2011). *C. gigas* has been farmed in France since the late 1970s, and the country is currently Europe's leading producer. Moreover, due to its bio-ecological characteristics, Pacific oyster is widely used as a bioindicator of water quality and a model species for marine ecotoxicology studies.

Marine organisms can be exposed to pollution by different routes. Depending on their bio-ecological characteristics (e.g. filter feeding, sessile) and on the properties of the pollutant itself (e.g. hydrophobicity), they can uptake pollutants from the sediment, the water column

(dissolved and particulate fraction) and food. The latter exposure route is particularly involved in the biomagnification of pollutants across trophic networks (Mackay et al., 2016, Xie et al., 2017). However, this route of pollutant exposure has not been as well investigated as the water column has. In the oyster, trophic transfer of chemical pollutants can occur via filter feeding activity of contaminated phytoplankton. Pollutants can in fact be adsorbed on and/or taken up by phytoplankton, as already demonstrated for PAHs (Wan et al., 2007), PCBs (Tiano et al., 2014) and trace metal elements such as Cu (Anu et al., 2016). As a consequence, trophic transfer of pollutants occurs in oysters by feeding on contaminated phytoplankton: in a recent study, Vilhena et al. (2016) calculated trophic transfer factors for different trace metal elements and highlighted differences in bioaccumulation efficiency in oysters. Phytoplankton can be a vector of contamination but remain a biological model of choice to assess the impacts of chemical contamination on both the structure and the function of marine ecosystems. Different ecological issues can be identified. As primary producers, microalgae sustain the development of higher trophic levels. Damage caused to microalgae in the natural environment could thus affect the quantity and quality of food available for primary consumers (Pennington and Scott, 2001, Gonçalves et al., 2017). Moreover, the trophic transfer of chemical contaminants can expose a wider range of organisms to pollution, away from chemical sources. By transferring chemicals and their potentially more toxic metabolism by-products, trophic transfer also promotes pollutant biomagnification through food webs to potentially toxic levels, endangering the diversity and the functioning of aquatic ecosystems (Walters et al., 2016).

In this context, the present study aimed to better assess the trophic transfer of Cu and its associated direct and indirect toxic effects, considering phytoplankton and oysters as the primary producer and the primary consumer, respectively. In some French areas supporting oyster production, Cu concentrations are still increasing in the different compartments of the environment including biota (Bachelot et al., 2009). Cu can have geochemical origin but increase in Cu concentrations in Arcachon Basin is believed to be mainly related to its use,

on the one hand, in antifouling paints since the ban on TBT (1982 in France) (Claisse and Alzieu, 1993) and, on the other hand, in some pesticide formulations (e.g. Bordelaise mixture in viticulture catchment). For these reasons, Cu is considered a pollutant of emerging concern in the Basin. The present study addressed the direct and indirect toxicity of Cu on phytoplankton and spat by an experimental approach. Its novelty is based on the diversity of the techniques used to measure chemical exposure and effects in both biota at the molecular, cellular and physiological levels. In addition to classical toxic endpoints, the impact of Cu exposure on the nutritional value of the phytoplankton used as oyster trophic source was also investigated in terms of the lipid composition. Data acquired during this study are hence valuable as trophic transfer of contaminants is generally assessed only from a chemical point of view and during field studies, due to the complexity to carry out such controlled experiments at the laboratory.

In the microalgae, we expected Cu effects at the highest exposure concentration on some gene expression levels linked to measured physiological endpoints (ROS intracellular level, lipid content), thus affecting the nutritional quality of microalgae. We also expected a bioaccumulation of Cu in spat together with sublethal effects on its growth and metabolism being consistent with the modulation of related target genes.

2. Materials and methods

The experiment run on oyster spat was split into two distinct phases (Figure 1). The first was a 2-week acclimation phase (Suppl. material), during which spat were prepared and maintained in experimental conditions close to the exposure conditions. This acclimation phase lasted from day -14 to 0. Then, there was a 3-week exposure phase, which consisted of feeding oyster spat with *Tetraselmis suecica* cultures previously grown under Cu contamination. This phase lasted from day 0 to 21.

2.1. Cu solution

The exposure experiment used a commercially available solution of Cu in its ionic form at 1,002 ± 3 µg.mL^{-1}; it had been prepared in 4% nitric acid (HNO_3) in ultra-pure water (SCP Science, Canada). Two working solutions, W1 and W2, were then made by dilution in ultra-pure sterile water for a final Cu concentration of 8,186 and 31,091 µg.L^{-1}, respectively. These working solutions were used to expose 5–8 L of *T. suecica* cultures to Cu concentrations of 12.5 and 47.5 µg.L^{-1}, respectively.

2.2. The marine microalga *T. suecica*

The marine microalga *T. suecica* CCMP 904 was obtained from the Provasoli–Guillard National Center for Marine Algae and Microbiota (NCMA). For the experiment, the microalga was cultured in sterile Conway medium (also called Walne's Medium; Walne, 1970; Andersen, 2005) with the following modifications. Ethylenediaminetetraacetic acid (EDTA) was completely removed to avoid Cu chelation and to ensure accurate Cu analysis by ICP-MS; in addition, considering the purpose of the study, the Cu concentration in the medium was lowered from 5 µg.L^{-1} (the initial concentration in Conway medium) to 2.5 µg.L^{-1} to bring sufficient micronutrients to the microalga in the control condition while limiting the Cu supply. Preliminary experiments showed that the microalga cultured without EDTA and at 2.5 µg.L^{-1} Cu (without bubbling) could grow sufficiently to feed oysters, reaching an exponential growth phase in two days after inoculation with a growth rate of 0.02 h^{-1}.

2.3. Culture of *T. suecica* in large volumes

All the cultures grown to perform the study, that is, pre-cultures and feeding experimental cultures, were maintained in a thermo-regulated room at 21 ± 1°C, under a light intensity of 260 ± 5 µmol m^{-2} s^{-1} (N = 117 measurements in the entire space dedicated to cultures; Quantometer Li-Cor Li-250 equipped with a spherical sensor), with a 8:16 dark:light cycle.

Pre-cultures were used to inoculate larger volumes, that is, feeding experimental cultures, which were further used to feed oyster spat during the exposure phase. Pre-cultures were grown in 100 mL to 6 L round borosilicate sterile glass flasks previously heated to 450°C for 6 h, autoclaved for 20 min at 121°C and then filled with 50 mL to 3 L of sterile culture medium. They were inoculated from mother cultures with an initial concentration of 100,000 cells.mL^{-1} and grown for about a week before inoculating the feeding experimental cultures. Mother cultures as well as pre-cultures were inoculated in sterile conditions by using a laminar flow cabinet to avoid contamination. Both pre-cultures and feeding experimental cultures were maintained under constant bubbling air enriched with a concentration of 1-2% carbon dioxide.

To provide phytoplankton for the entire exposure phase, feeding experimental cultures were inoculated at an initial concentration of 100,000 cells.mL^{-1} using pre-cultures. They were grown in larger volumes from 5 to 8 L, in single-use 150-µm-thick low-density polyethylene thermo-weldable sheath. Before inoculation, the sheath was cut at the right height for the targeted culture volume and it was thermo-welded. Next, it was filled with the precise volume of seawater, to which a sodium hypochlorite solution was added at a final concentration of 0.1 mL.L^{-1} (48–55° chlorine) and the sheath was left to disinfect for 4 h under bubbling air to facilitate homogenisation. Then, a 350 g.L^{-1} sodium thiosulfate solution was added at a final concentration of 0.1 mL.L^{-1} to neutralise the chlorine; the solution was incubated for 30 min. Once disinfected and neutralised, the sheath was ready to start a culture. Modified Conway enrichment medium (containing 2.5 µg Cu.L^{-1}) was added to the seawater together with the required amount of Cu: either no Cu added (control condition) or the addition of an appropriate volume of W1 or W2 solutions to reach the targeted concentrations of 15 or 50 µg Cu.L^{-1}, respectively. Finally, inoculum was added. Feeding experimental cultures were started as series, with each one corresponding to a set of three conditions: one control culture (C), a second culture grown under 15 µg Cu.L^{-1} (Cu15) and a third one grown under 50 µg Cu.L^{-1} (Cu50). After 7–9 days of growth, each series was used over 2 consecutive

days to feed the oyster spat in the tanks corresponding to the respective exposure conditions; 11 series (S1–S11) were started over the 3-week exposure phase. The culture volume of each series was adjusted depending on the number of remaining spat to feed as the sampling progressed over the experiment.

2.4. The *C. gigas* oyster spat

Spat from *C. gigas* used for this study came from a cohort produced in December 2017 at the experimental hatchery of Ifremer Argenton (France). They were considered to be naive, being produced following a standardised protocol based on the production of OsHV-1-free diploid oysters (Petton et al., 2013). They arrived at the experimental facilities of Ifremer Bouin in March 2018. At this time, spat were 16.75 ± 0.63 mm length (mean ± standard error [SE], N=4), 0.85 ± 0.04 g total weight and 0.13 ± 0.04 g total wet flesh weight. They grew up in a controlled hatchery up to middle May for the beginning of the acclimation (the individuals were then 5-month old). Following the ongoing procedures in the hatchery, oysters were fed with a multispecific regimen mainly composed of *Skeletonema marinoi* grown in outdoor ponds, from their arrival until the beginning of acclimation.

At the beginning of the acclimation period which lasted 14 days (see supplementary material), oyster spat were distributed into nine high-density polyethylene tanks (570 × 370 × 265 mm [inner dimensions], 56 L), three for each experimental group (C, Cu15 and Cu50), in a temperature-controlled room (17°C). A 600-L stock tank containing seawater directly pumped from the Bourgneuf Bay, filtered at 1-µm filtration (Bubba cartridge) and UV-sterilized (1160 HO, 40 mJ.cm^{-2}), was used to fill each experimental tank. As the experimental tank water was renewed every day, the stock tank was filled each day and left 24 h to reach the target temperature of 17°C, by means of a circulating thermostat device. The physicochemical parameters of the water such as dissolved oxygen saturation, pH, salinity and temperature were measured regularly during the acclimation (before and after

the addition of microalgae) period using a WTW FDO 925 probe (dissolved oxygen), a WTW Sentix 940-3 probe (pH) and a WTW cond 325 probe (salinity and temperature). Their values (mean ± SE) were 7.69 ± 0.02 mg.L^{-1} (dissolved oxygen), 7.59 ± 0.02 (pH), 32.53 ± 0.05 (salinity) and 17.48 ± 0.14°C (temperature). During the 2-week acclimation period, the volume of water provided per individual was maintained at 172 mL and water was renewed every morning just after tank cleaning and before oyster feeding.

2.5. Cu trophic exposure of oyster spat

After 14 days of acclimation, the trophic exposure experiment started (day 0). Oyster spat were fed using the experimental feeding cultures from the three previously described conditions: C (containing 2.5 µg Cu.L^{-1}), Cu15 (containing 15 [2.5 + 12.5] µg Cu.L^{-1}) and Cu 50 (containing 50 [2.5 + 47.5] µg Cu.L^{-1}).

2.5.1. Measurement of culture concentration

Each experimental culture was sampled in the morning (1.5 mL) and its cell density measured by flow cytometry using a Partec CyFlow Space cytometer equipped with a blue laser (488 nm emission). Due to the technical limits of the cytometer, cultures were diluted, using sterile seawater, to a target concentration of 1 x 10^5 to 5 x 10^5 cells.mL^{-1} to ensure a reliable measurement of the concentration. Diluted cultures were fixed using glutaraldehyde at a final concentration of 0.25%, vortexed and left for 15 min in the dark at room temperature before analysis. A 850-µL sample of diluted culture was analysed. Cells were discriminated against and counted on an FL1 (536/540 nm) versus FL3 (> 675 nm) cytogram. The absolute counting and measurement of the analysed volume provided a direct determination of the cell density of the diluted cultures and, consequently, the experimental cultures.

2.5.2. Microalgal distribution for oyster feeding

Similarly to the acclimation period, oysters were fed once a day with the same target microalgae ration of 5.8 × 10^6 cells per spat. Cell density measurements were performed to

calculate the right volume of each experimental culture to sample in order to feed spat contained in each tank of the corresponding treatment for 2 days (N = 3 tanks per treatment). Each culture was sampled using a 50-mL sterile pipette and put into a sterile flask culture, before being poured into the corresponding tanks. As cells could not be separated efficiently from the culture medium, it was chosen to distribute the cell suspensions into the oyster tanks. Consequently, the oysters were exposed to Cu due to their trophic resource as well as the dissolved Cu contained in the culture medium. This source of Cu contamination was quantified during the experiment by the use of passive samplers (see section 2.8.1). After food distribution and before the oysters were put back into the tanks, microalgae were left for a few minutes to homogenise in the tanks. Each tank was then sampled three times to check for the cell density available for the spat, using flow cytometry measurements. During the entire experiment, the mean microalgae ration distributed daily was 5.96 ± 0.02 × 10^6 cells per spat (mean ± SE, N = 339), corresponding to 0.998 mg dry weight (d.w.).$spat^{-1}$.day^{-1}.

2.6. Verification of *T. suecica* Cu exposure concentration

2.6.1. Dissolved Cu concentration in the culture medium from the different conditions

Four culture series (S2, S4, S5 and S8) out of the 11 produced during the experiment were sampled to check whether Cu exposure concentrations were close to the nominal targeted Cu concentrations of 2.5, 15.0 and 50.0 µg.L^{-1}. For each series and culture condition, 2.5 mL of culture medium was sampled in triplicate at day 0 just before inoculating phytoplankton. Samples were first diluted (1/20) to reduce their salt concentration. Briefly, they were acidified with 3 mL of ultrapure HNO_3 (69-70%) and the volume was adjusted to 50 mL with ultrapure Milli-Q water. Cu concentrations were then determined with a quadrupole inductively coupled plasma mass spectrometer (Q-ICP-MS, model ICAP-Qc, Thermo Fisher Scientific), according to a calibration range varying from 0 to 4 µg.L^{-1}. To maintain the same salt conditions as the samples, metal-free ‘cleaned’ seawater was used to prepare the

calibration range. This metal-free 'cleaned' seawater was derived from internal analyses of natural seawater, according to a protocol adapted from Danielsson et al. (1982) that involves a liquid/liquid extraction procedure of trace metals in seawater. Furthermore, a known quantity of internal standard was added to each sample (before adjusting the volume to 50 mL) as well as in the calibration range to check (and to correct if necessary) for any drift of the mass spectrometer during measurements. The concentrations determined ($\mu g.L^{-1}$) were finally corrected for blank controls and for the dilution factor (1/20).

2.6.2. Cu concentrations in *T. suecica* pellets

For S2, S4, S5 and S8 series, sampling occurred (i) on day 0 for the pre-culture used to inoculate a culture series and (ii) on day 7 and 8 for each experimental culture used for oyster feeding. Depending on the cell concentration, a volume of 40–260 mL could be filtered until clogging through 1-µm mesh pre-cleaned with ultrapure HNO_3 acid and pre-weighed polycarbonate filters (Nucleopore®) under a clean laminar flow hood. Filtration was realised under gentle pressure to avoid cell disruption. Filters were then frozen at -20°C, dried at 50°C in an oven overnight and weighed again to obtain the phytoplankton mass (in mg d.w.) on each filter. Dried filters were placed in microwave Teflon bombs and mineralised at 105°C for 4 h using a mixture of 3 mL of ultrapure HNO_3 (69-70%) and 3 mL of Milli-Q water. The volume of the digests were then adjusted to 50 mL with Milli-Q water. The total Cu concentrations in these samples were finally determined using Q-ICP-MS, according to a calibration range from 0 to 40 $\mu g.L^{-1}$. Regarding dissolved Cu concentrations in the phytoplankton culture medium, a known quantity of internal standard was added to each sample (before adjusting the volume to 50 mL) as well as in the calibration range to check (and to correct if necessary) for any drift during measurements. Moreover, the quality assurance of all metal analyses relied on blank controls as well as the accuracy and reproducibility of data relative to the concomitant measurement of a certified reference material (CRM) in each analytical run. The CRM used was BCR-414 (plankton, Joint Research Centre- European Commission). CRM values concurred with the certified

concentrations, with recovery rates ranging between 82% and 92%. The concentrations determined (µg.L^{-1}) were corrected for blank controls and converted to mg.kg d.w.$^{-1}$ and fg.cell^{-1} according to the mass of phytoplankton on each filter (in mg d.w.), the mean cell mass and the recovery volume of the digest (50 mL).

2.7. Biological effects of Cu exposure on *T. suecica*

2.7.1. Growth rate

The daily measurement of cell density (section 2.6.1) for each condition in each culture series made it possible to calculate their growth rates over the 7–9 days of culturing. For each culture, the growth rate was calculated over the culture duration with the equation:

$\mu = \ln(C_t - C_0)/t$,

where C_t is the cell density (cells.mL^{-1}) of the culture at t (in days), µ (days^{-1}) is the growth rate and C_0 is the initial cell density at t = 0 days (Andersen, 2005).

2.7.2. Physiological endpoints

In addition to the growth rate, the possible effects of Cu in *T. suecica* cells were assessed using various fluorescent dyes and flow cytometry (Partec CyFlow Space cytometer equipped with a blue laser with emission at 488 nm). Cells were stained to examine the presence of reactive oxygen species (ROS) inside the cells, using the dyes 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA or DCFH-DA, Sigma-Aldrich) and dihydroethidium (or hydroethidine [HE], Thermo Fisher Invitrogen). The green lipophilic fluorochrome BODIPY$^{505/515}$ (Thermo Fisher Invitrogen) was used to estimate the relative intracellular lipid content. The fluorescein-based lipophilic dye fluorescein diacetate (FDA, Thermo Fisher Invitrogen) was used to highlight the metabolic activity of the cells and its possible changes under Cu exposure. Finally, cells were also stained with the slow-response

potential-sensitive probe $DiBAC_4(3)$ (Thermo Fisher Invitrogen) to check their cytoplasmic membrane potential.

Staining was performed each day (except during weekends) on cultures diluted in the same way as for growth measurements. Each staining was performed with 850-µL samples of cultures from working solutions in pure dimethyl sulfoxide (DMSO). Detailed information regarding the staining protocols are presented in supplementary Table S1. The staining protocols were from Stachowski-Haberkorn et al. (2013) for DCFH-DA, from Coquillé et al. (2018) for $BODIPY^{505/515}$, from Prado et al. (2012) for HE and $DiBAC_4(3)$ and adapted from Dupraz et al. (2019) for FDA. In addition to dyes, high-intensity green fluorescent beads (Polysciences Europe) were added to each tube (8.5 µL per tube) to permit a normalisation of the fluorescence values of cells for further comparisons among samples and over time. For each target, the normalisation was determined with the formula:

$$FL_{target} = (FL_{stained\ microalgae}/FL_{stained\ beads})/(FL_{unstained\ microalgae}/FL_{unstained\ beads}).$$

2.7.3. Lipid analyses

The lipid analyses were performed by the LIPIDOCEAN platform (LEMAR, Brest, France). They allowed the characterization of lipid class, FA and sterol composition on three series of phytoplankton cultures (S5, S8, S9) out of the 11 produced during the experiment. For each series, a volume of culture was sampled at day 7 and 8 of culture growth. This volume was calculated based on the algal concentration to provide an approximate quantity of 2.7×10^8 cells for the analysis (about 45 mg d.w.). Each culture sample was filtered on GF/F 0.7-µm glass microfiber filters with a 47 mm diameter previously heat cleaned (6 h at 450°C). Immediately after the end of the filtration, 50 mL of boiling distilled water was poured on each filter to deactivate lipases. Filters were then put in 6 mL of chloroform/methanol (2:1, v/v) and stored at -20°C under a nitrogen atmosphere to avoid oxidation. Details on lipid analyses are available in supplementary material.

2.7.4. Target gene expression by quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

The expression of 28 target genes was measured for each culture series S2, S4, S5, S8, S9 and S11 (supplementary Table S2). Around 3 × 10^7 cells of *T. suecica* (corresponding to 5 mg d.w.) were sampled in C, Cu15 and Cu50 conditions on each feeding day. Cultures were filtered on 0.2-µm polycarbonate membranes (Whatman®), then the membranes were washed with 1.5 mL of TRIzol (Ambion, Life Technologies) and stored at -80°C until RNA extraction according to the manufacturer's instructions. The RNA concentration was determined using an ND-1000 spectrophotometer (Thermo Scientific, Waltham MA, USA) at 260 nm. RNA was treated with DNase I (0.1 U per µg of RNA, Ambion, Life Technologies) following the manufacturer's instructions and precipitated with isopropanol (v/v) and 0.3 M sodium acetate. The RNA integrity was assessed on an Agilent bioanalyser using RNA 6000 Nano kits (Agilent Technologies, Santa Clara, CA, USA). Reverse transcription to complementary DNA (cDNA) was carried out on 1 µg of total RNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies) according to the manufacturer's instructions. Primers were designed on conserved sequences from a RNA-seq experiment previously conducted in the laboratory (data not published) using Primer 3 V 4.0.0 (Untergasser et al., 2012). The primers used and the associated information are available in Table S2. The PCR efficiency (E) was assessed for each primer on serial dilutions of a pooled sample containing cDNA from all experimental conditions. PCR was carried out in triplicate in 96-well microplates. Each reaction had a final volume of 20 µL and contained Agilent Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix, 0.5 µM of each primer and cDNA diluted to 1/40. Accurate amplification of the target amplicon was checked by performing a melting curve. Each run included a cDNA inter-run control (composed of a pooled sample containing cDNA from all experimental conditions), a no-template control and a water control. The geometric mean of the three reference genes was used to normalise gene expression using the $2^{-\Delta\Delta Ct}$ (Livak) method (Livak and Schmittgen, 2001).

A summary of the sampling performed in *T. suecica* cultures during the duration of the experiment is provided in supplementary Table S3.

2.8. Sampling program for chemical and biological analysis of oyster spat

The exposure phase started after the end of the acclimation phase (day 0). Sampling occurred on days 0 (start of the experiment), 2, 7, 14 and 21. At each time, spat were sampled in each tank to measure selected chemical and biological markers in triplicate. The number of individuals sampled for each analysis is summarised in supplementary Table S4.

For the spat Cu concentration, 14 juvenile oysters were sampled from each tank (N = 3 per treatment). They were maintained for an additional 24 h in clean seawater without feeding in the same conditions as described previously to allow the elimination of undigested particles. After depuration, whole oyster tissues were collected and pooled in 69-mL polypropylene tubes (SYMALAB). Each tube was then frozen at -20°C until it was used for analysis.

In each tank, 30 juvenile oysters were also sampled for gene expression analysis, enzymatic activities, the glycogen content and lipid peroxidation measurement. The flesh of these animals was collected and pooled in three batches of 10 individuals on a calcined aluminium pan and immediately frozen in liquid nitrogen. Each batch of frozen tissue was then ground into a fine powder at -196°C with an MM400mixer mill (Retsch) and the powder was aliquoted and directly stored at -196 or -80°C (according to the intended analysis) prior to use.

Three individuals were also taken from each tank to sample haemocytes for the comet assay. The haemolymph from each individual was collected by aspiration directly from the heart using a sterile hypodermic needle (23 G) in a 2-mL syringe pre-rinsed with the anticoagulant Alsever's solution (113.7 mM glucose, 27.2 mM sodium citrate, 58.4 mM sodium chloride, pH 6.1). The haemolymph from the three individuals was pooled in a

microtube and then centrifuged for 5 min at 1,500 rpm to recover the haemocyte pellet. All pellets were stored on ice and in the dark before proceeding with the comet assay.

Finally, non-destructive biometry measurements were taken on the 30 identified animals. At each date, the length, width and total weight were measured. At the end of the experiment (day 21), the animals were dissected and quickly wiped off to weigh the shell and the wet flesh. The wet flesh was then dried at 60°C for 72 h to obtain the dry flesh weight.

2.8.1.Contribution of labile Cu to spat exposure

The Cu concentration in seawater from the hatchery was measured using diffusive gradient in thin film (DGT) passive samplers (Zhang and Davison 1995). DGT concentrations ('labile' concentrations) correspond to the concentrations of metal cations in the form of hydrated ions, mineral complexes and 'small' organic complexes. DGT were also used to better assess the contribution of the different forms of Cu (dissolved and particulate) to spat exposure. Indeed, it is necessary to note the possible contribution of Cu to spat contamination from the small volume of phytoplankton culture medium spilled into the tanks when feeding the oysters. Details on DGT deployment and analysis are supplied in supplementary material.

2.8.2. Cu concentration in spat

Each spat replicate was digested with HNO_3. Specifically, between 80 and 110 mg of the dry tissue was collected in polypropylene tubes and 3 mL of HNO_3 was added. Then, this mixture was heated in a hot block for 3 h at 100°C. During that time, eight tubes containing approximately 90 mg d.w. of the international CRM DOLT-5 (fish liver) were prepared to verify the accuracy of the method. Eight blank samples were also prepared. After cooling, the samples were diluted with 15 mL of ultrapure water (Milli-Q®).

Cu was analysed by inductively coupled plasma optical spectrometry (ICP-OES, Agilent Technologies, 700 Series) as described in Machavaram (2011).

The mean Cu concentration (33.6 ± 0.5 $mg.kg^{-1}$) in the CRM was within the certified range (35.0 ± 2.4 $mg.kg^{-1}$).

2.8.3. Condition index (Ci)

By using raw biometry data, the Ci described by Lawrence and Scott (1982) was calculated as follows:

dry flesh weight / (total weight– shell weight)] x 1000.

2.8.4. Glycogen content

Glycogen was extracted using trichloroacetic acid (TCA) as described by Timmins-Schiffman et al. (2014). For each individual sample, 3 mL of 15% TCA was added to approximately 100 mg of frozen tissue powder (see section 2.9). Following homogenisation by mechanical grinding (Potter-Elvejhem tissue homogenisers), samples were stored at 4°C for 1 h. Samples were then centrifuged at 3,000 *g* for 10 min at 4°C and the supernatants were recovered and mixed with 12 mL of absolute ethanol. Following overnight incubation at 4°C under gentle shaking, samples were centrifuged at 4,000 *g* for 30 min at 4°C to recover the glycogen pellets. Once dissolved in 600 µL ultra-pure water, glycogen was quantified using the procedure described by Laurentin and Edwards (2003). Briefly, 40 µL of diluted sample was added to individual wells of a microtitration plate (each sample was run in triplicate). This plate was covered with cling film mixed gently and incubated at 4°C for 15 min. Then, 100 µL of anthrone solution (2 $g.L^{-1}$ in concentrated sulfuric acid) was added to each well. The plate was sealed with adhesive foil and mixed gently before incubation at 92°C for 3 min in a non-shaking water bath. The reaction was stopped by incubation for 5 min at room temperature and dried for 15 min in an oven at 45°C. The optical density was measured on a microplate

reader (Safire TECAN) at 630 nm. Using purified oyster glycogen as a standard, it was possible to measure the concentration of each sample in mg glycogen.mg wet weight^{-1}.

2.8.5. Lipid peroxidation

Under oxidative stress, PUFA peroxides can generate malondialdehyde (MDA), which is widely used as an indicator of lipid peroxidation (Pampanin et al., 2005). The determination method used was designed to measure thiobarbituric acid reactive substances (TBARS), including MDA, following the protocol of Buege and Aust (1978) adapted for a microplate. Fifteen milligrams of spat powder were homogenised in 800 µL of phosphate buffer (0.1 M, pH 7.5, 4°C) and centrifuged at 9,000 *g* for 20 min at 4°C. The supernatant fractions (S9) were separated in two tubes for TBARS (200 µL) and total protein (50 µL). For TBARS, 100 µL of a solution containing 2% butylated hydroxytoluene and 20% TCA (1:200) was added to 200 µL of S9. The mixture was then centrifuged at 10,000 *g* for 10 min and 200 µL of supernatant was added to 160 µL of a solution containing 25 mM Tris base (25 mM) and 100 mM thiobarbituric acid (TBA), and 40 µL of 0.6 N HCl; the mixture was heated at 80°C for 15 min. The blends were then cooled and mixed before being distributed in a microplate. The TBARS levels were then read using a UV spectrophotometer (Synergy HT, BioTek) at 530 nm and the results are expressed as nmol TBARS equivalents.mg protein^{-1}.

The total protein concentration was measured using the Lowry method (Lowry et al., 1951) with bovine serum albumin as the standard. Measurements were performed in a spectrophotometer microplate reader (Synergy HT) and protein concentrations are expressed as mg.mL^{-1}.

2.8.6. Catalase (CAT) activity

The CAT activity was measured in the subcellular S9 fraction. For each sample, this subcellular fraction was obtained by resuspension of 200 mg of spat powder in 600 µL of Tris buffer (50 mM Tris, 0.3 M sucrose, 1 mM EDTA, pH 7.4) and centrifugation at 9,000 *g* for 20

min at 4°C. The supernatant containing S9 proteins was recovered and a small fraction was used to determine the protein concentration with the Bradford method (Bradford, 1976), the basis for the Bio-Rad Protein Assay Dye Reagent Concentrate, using bovine serum albumin as a standard.

The CAT activity was measured by spectrophotometry as described by Clairborne (1985) and adapted for a microplate. This method is based on the decrease in absorbance at 240 nm due to hydrogen peroxide (H_2O_2) consumption. For each sample, the CAT activity contained in 5 µg of S9 proteins was measured in triplicate. The reaction started just after the addition of 80 mM H_2O_2 solution. The absorbance was then read every 60s over a period of 5 min at 25°C by using a microplate reader (Safire TECAN). The CAT activity is expressed as µmol H_2O_2 degraded.min^{-1}.mg protein^{-1}.

2.8.7. DNA strand breaks

For each pooled sample, the haemocyte pellet was resuspended in 0.5% low-melting-point agarose. Following resuspension, two comet slides were prepared by depositing 75 µL of this mixture on each of the pre-coated agarose slides. A final layer of 0.5% normal-melting-point agarose was deposited on each slide prior to performing the lysis, denaturation and electrophoresis steps as described previously (Barranger et al., 2013). Just prior to reading the slide, 75 µL of CelRed (8 mg.L^{-1}) was spread over each slide using a cover glass. The slides were placed for at least 1 h in the dark at 4°C for colouration and then analysed using an optical fluorescence microscope (Olympus BX60, 40× objective lens) equipped with a CDD camera (Luca-S, Andor Technology) and an image analysis system (Komet 6, Kinetic Imaging Ltd.). On each slide, 100 nuclei were analysed for their percentage of DNA present in the comet tail (% tail DNA).

2.8.8. Target gene expression by RT-qPCR

The primer sequences and Genbank accession numbers for the 73 genes considered are in supplementary Table S2. Total RNA was isolated from frozen tissue powders using TRIzol Reagent according to the manufacturer's instructions. RNA concentrations were determined using an ND-1000 spectrophotometer. RNA was treated with DNase I (as described in section 2.8) and precipitated with isopropanol (v/v) and 0.3 M sodium acetate. RNA integrity was assessed on an Agilent bioanalyser using RNA 6000 Nano kits, according to the manufacturer's instructions. Reverse transcription was carried out on 1 µg of total RNA with the High-Capacity cDNA Reverse Transcription Kit, according to the manufacturer's instructions. For each candidate gene, specific primers were designed using Primer 3V 4.0.0 (Untergasser et al., 2012) (supplementary Table S2). Real-time PCR was performed with the Biomark HD System (Fluidigm, France) using the GeT-PlaGe genotyping service platform (INRA, Toulouse, France) according to the manufacturer's recommendations. Accurate amplification of the target amplicon was checked by performing a melting curve. The geometric mean of four reference genes was used to normalise gene expression using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

The different statistical analyses performed are summarised in Table 1.

3. Results

In the whole section, means are given with their respective standard errors (SE).

3.1. Cu concentrations in control and contaminated culture medium

Chemical analysis of the dissolved Cu in the culture medium, before the addition of the microalgal inoculum, showed concentrations very close to the ones targeted: 3.1 ± 0.1 $\mu g.L^{-1}$, 15.7 ± 0.2 $\mu g.L^{-1}$ and 50.4 ± 1.0 $\mu g.L^{-1}$ for the control (2.5 $\mu g.L^{-1}$ target), Cu15 (15 $\mu g.L^{-1}$ target) and Cu50 (50 μgL^{-1} target) conditions, respectively.

3.2. Cu concentrations in control and exposed phytoplankton pellets

Just before inoculation of a series of experimental cultures from a single pre-culture, the Cu concentration found in the microalgal pellets was 1.1 ± 0.2 fg Cu.cell^{-1}, corresponding to 6.9 ± 1.2 mg Cu.kg d.w.$^{-1}$. In the microalgal pellets from the control condition, the Cu concentration did not significantly evolve over time until day 8. In the Cu-exposed conditions, Cu accumulated significantly in the microalgal pellets from day 0 to 7: by 3 fold in Cu15 cultures, reaching 3.3 ± 0.6 fg Cu.cell^{-1} (28.3 ± 0.9 mg Cu.kg d.w.$^{-1}$), and by 15 fold in Cu50 cultures, reaching 16.7 ± 1.2 fg Cu.cell^{-1} (110.7 ± 11.9 mg Cu.kg d.w.$^{-1}$). There were no significant differences in Cu concentrations of the microalgal pellets between day 7 and 8 for the three conditions.

3.3. Biological effects of Cu exposure on *T. suecica*

3.3.1. Growth and physiological endpoints

The growth rate and physiological parameters were determined on 7- and 8-day-old cultures used for oyster feeding (except during the weekends). As shown in the PCA, the first two axes explained around 65% of the total variance (Figure 2) with a poor representation of the growth rate. The first axis, which explained most of the total variance, illustrated a global separation between most control and Cu-treated cultures (Figure 2A). As shown by the variable map, the former, grouped on the right-hand side, were distinguished from the latter by their overall higher relative lipid content and metabolic activity (Figure 2B). Cu-treated cultures on the left side were distinguished by their overall higher ROS content, measured via HE and DCFH-DA. The second axis, which represents $DiBAC_4(3)$ (uncorrelated to FDA, $BODIPY^{505/515}$ and DCFH), did not differentiate between conditions, days or series.

3.3.2. Lipid, sterol and FA content

3.3.2.1 Neutral Lipids

AL were not detected in phytoplankton regardless of the culture series analysed and the experimental group (supplementary Table S5). GE were detected in S5 cultures but only in those exposed to Cu. The opposite was observed in S9 cultures, with GE only detected in the control culture at day 8. There was a different pattern for S8 cultures: whereas GE were detected in the control culture at day 7 and 8, it was only detected at day 7 in the cultures exposed to Cu. TG (62.1% ± 10.5%) and FS (25.7% ± 8.2%) represented more than 80% of NL present in phytoplankton.

3.3.2.2 Polar Lipids

During this experiment, SPG was not detected in the control and Cu-exposed phytoplankton cultures (supplementary Table S6). LPC was not detected in the S9 control culture on day 7. PC was the most abundant PL (21.9% ± 2.2%), followed by PE+PG+SQDG (19.2% ± 1.8%), DGDG (17.7% ± 3.3%), CI (16.6% ± 1.5%) and MGDG (15.1% ± 2.1%).

3.3.2.3 Sterols

Cholestone remained undetected in control and Cu-exposed cultures (supplementary Table S7). Stigmasterol was weakly detected only in the C and C50 S5 cultures. Campesterol and ergosterol constituted up to 65.2% ± 3.4% and 34.0% ± 2.8%, respectively, of the sterols present in phytoplankton.

3.3.2.4 Fatty Acids

Several types of FA were detected in phytoplankton, including branched chain fatty acids (BCFA: 4,8,12-trimethyltridecanoic acid, iso15:0, ante15:0, iso16:0, iso17:0), saturated fatty acids (SFA: 14:0, 15:0, 16:0, 17:0, 18:0, 20:0), monounsaturated fatty acids (MUFA: 16:1n-9, 16:1n-7, 16:1n-5, 18:1n-9, 18:1n-7, 20:1n-9, 20:1n-7) and polyunsaturated fatty acids (PUFA

(16:2n-7, 16:2n-6, 16:3n-4, 16:3n-6, 16:3n-3, 16:4n-3, 16:4n-1, 18:2n-6, 18:3n-6, 18:3n-3, 18:4n-3, 20:2n-6, 20:3n-6, 20:3n-3, 20:4n-6, 20:4n-3, 20:5n-3, 22:2n-6, 22:5n-6, 22:5n-3, 22:6n-3) (supplementary Table S8). Omega 3 (n-3: 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, 22:6n-3) and omega 6 (n-6: 16:2n-6, 16:3n-6, 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:2n-6, 22:5n-6) constituted the bulk of PUFA. Overall, PUFA represented 35.7% ± 1.0% of FA, of which 61.0% ± 1.7% were n-3. They were followed by SFA and MUFA, representing respectively 21.7% ± 0.5% and 19.3% ± 2.1% of the total FA present in phytoplankton.

A PCA performed on the sum of NL, the sum of PL, the sum of sterols and the sum of all FA as variables, made it possible to distinguish globally the cultures of day 7 and day 8. On day 8, the cultures were richer in all lipid classes than on day 7, whether they were control cultures or those exposed to Cu (see supplementary material Figure S1). Complementary PCA performed on day 7 and 8 separately did not show any distinction between cultures considering either the series or the treatment (see supplementary material).

For FA, an additional PCA analysis was performed (Figure 3). Because more than 44 individual FA were quantified, the data were first graphically represented to select some of those as variables for the analysis. PCA was then performed considering the BCFA ante 15:0, the NUFA 16:1n-5, the SFA 15:0 and 20:0, the PUFA 22:6n-3 and the sum of other quantified FA (six variables). The 18 cultures were identified as observations. Pearson's correlation matrix showed that SFA 15:0 and 20:0 were positively correlated to the sum of remaining FA whereas the others were not (supplementary Table S11). The BCFA ante 15:0 was positively correlated to SFA 15:0 and MUFA 16:1n-5 whereas it was negatively correlated to PUFA 22:6n-3. The latter FA showed an opposite trend to that observed for the BCFA, SFA and MUFA selected for the analysis, being negatively correlated to BCFA ante 15:0. The first two factors, F1 and F2, explained 72.62% of the data dispersion. The biplot of observations and variables showed that the Cu-exposed cultures presented higher contents

of SFA 15:0 and 20:0, BCFA ante 15:0 and MUFA 16:1n-5. On the contrary, Cu-exposed cultures had less PUFA 22:6n-3 compared with the control cultures (Figure 3).

3.3.3. Target gene expression

In the PCA performed on gene expression data from *T. suecica* exposed to Cu, the variance was not explained by the experimental conditions (Cu exposed vs control) or by growth duration (day 7 vs 8) (Figure 4). Complementary PCA were also realised separately for day 7 and 8 data (data not shown) but gene expression variance was also not explained by the experimental conditions. Each culture series had a singular gene expression pattern, making it impossible to infer gene expression regulation mechanisms due to Cu exposure.

3.4. Cu transfer from phytoplankton to oyster spat tissue

At the beginning of the exposure phase (day 0), the Cu concentrations in spat were similar among the experimental units, with a mean value of 139.2 ± 13.0 mg.kg^{-1} d.w. (Figure 5). In the C condition, the Cu concentration decreased by 36% between days 0 and 7 and reached a final concentration of 74.68 ± 13.24 mg.kg^{-1} d.w. In the Cu15 condition, the Cu concentration followed the same pattern as in the C group. However, from day 7, the Cu concentration was higher than in the C condition and stabilised around 104.9 ± 11.0 mg.kg^{-1} d.w. For the Cu50 condition, there was a general decrease in the Cu concentration, but it followed an irregular pattern. From days 0 to 7, there was a decrease in the Cu concentration in spat, followed by an increase between days 7 and 14. At day 21, the concentration for this condition (124.1 ± 10.3 mg.kg^{-1} d.w.) was close to concentration measured at the beginning of the exposure experiment, although significantly higher than in the C and Cu15 conditions.

Cu uptake occurred mostly from the absorption of contaminated phytoplankton. As a matter of fact, the use of DGT in each experimental unit of the different exposure conditions showed the absence of significant differences in the 'labile' Cu concentrations between the C, Cu15 and Cu50 conditions (one-way ANOVA, $p = 0.205$) (supplementary Figure S2).

3.5. Biological effects in spat fed with Cu-exposed phytoplankton

3.5.1. Condition index

At the end of the 21-day exposure experiment, the Ci values from each experimental group showed no significant differences between triplicates. Nevertheless, there was a significant decrease in the growth of juvenile oysters when they were fed with phytoplankton exposed to 50 $\mu g.L^{-1}$ Cu (Tukey's test, $p < 0.001$) (Figure 6, $p = 0.002$).

3.5.2. Glycogen content

There was no significant difference in the glycogen content between oysters from the control and Cu-exposed groups (Main effects ANOVA, $p = 0.039$). Whatever the feeding conditions, a significant decrease in the glycogen content was observed over time (Main effects ANOVA, $p < 0.001$) (Figure 7). Decrease was continuous and significant, except between day 7 and day 14 (Tukey's test, $p = 0.168$).

3.5.3. Lipid peroxidation

A significant decrease in TBARS was evidenced between day 0 (8.05 ± 1.03 nmol. mg^{-1} protein) and day 14 (2.40 ± 0.24) in the control group. Under Cu exposure, a significant decrease was also observed between day 0 (12.46 ± 2.03) and day 2 (2.92 ± 0.41) in the Cu15 group and between day 0 (12.72 ± 2.42) and day 7 (4.16 ± 0.47) in the Cu50 group.

Whether over the entire duration of the exposure experiment or at a given sampling time, no differences in TBARs were observed between the control and Cu-exposed conditions ($p > 0.05$).

3.5.4. CAT activity

There was no difference in the CAT activity in spat, whether they were fed with control or Cu-exposed phytoplankton cultures (main effect ANOVA, $p > 0.05$). The CAT activity during the 21-day period was 7.43 ± 0.77, 7.53 ± 0.68 and 8.16 ± 0.70 $\mu mol.min^{-1}.mg^{-1}$ for C, Cu15 and Cu50, respectively.

3.5.5. DNA damage

The main effect ANOVA showed no difference in the extent of DNA strand breaks among the three experimental groups ($p = 0.11$). The mean value of percentage DNA in the comet tail of spat haemocytes was 12.19% ± 0.32%, 13.45% ± 0.35% and 12.89% ± 0.33% when fed control, Cu15 and Cu50 phytoplankton cultures, respectively. There was a slight but significant increase in DNA breakage over time ($p < 0.001$).

3.5.6. Target gene expression

Target gene expression of oysters showed 18 of 73 evaluated genes exhibited a significant difference due to time or condition ($p < 0.05$, N = 3) according to the nested ANOVA. These genes code for the following functions: autophagy (*atg8*), apoptosis (*caspase 9*), DNA repair (*gadd45, xrcc4*), the stress response (*cox2, hsp70, mt1* and *mt2*), Cu transport (*copper transporter 2*), xenobiotic metabolism (*cyp1a1*), DNA methylation (*cxxc1*), fatty acid oxidation (*adipr1*, *ptl2*), ATP synthesis (*as6*), glycogen catabolism (*gly phos*), energy metabolism (*npyr*), mitochondrial metabolism (*vdac2*) and development (*nr8*). A heatmap (Figure 8) of these genes highlights patterns of expression, namely overexpression in the Cu15/Cu50 groups compared with the control group on days 14 and 21, particularly for genes involved in the stress response, DNA repair, glycogen catabolism and FA metabolism. A boxplot of

genes presenting expression differences with the post hoc test results are presented in Figure S3.

4. Discussion

4.1. *T. suecica* pellets display Cu concentrations close to those measured in natural particulate organic matter

By exposing *T. suecica* to Cu, we reached concentrations in phytoplankton (28.3 and 110.7 mg kg^{-1} d.w. for Cu15 and Cu50, respectively) that are the same order of magnitude as those reported in plankton (phytoplankton-zooplankton, 6–200 µm) from the Gulf of Lion in the north-western Mediterranean Sea (Chouvelon et al., 2019). In the latter study, Cu concentrations reached 58.8 ± 11.1 and 30.0 ± 11.6 mg kg^{-1} d.w. in the smallest size fraction (6–60 µm) and to a lesser extent in the 60–200-µm fraction respectively. Similar Cu concentrations were also measured in another recent study conducted in the Arcachon Bay (French Atlantic coast) (unpublished results). Cu concentrations were determined in the 6–100-µm fraction with values ranging from 25-380 to 700 mg kg^{-1} d.w. depending on the tide, sampling site and season. These high concentrations are in the same range as those measured in other seas, such as in the Azov Sea (Eastern Europe), with values between 48 and 93 mg kg^{-1} d.w. in total phytoplankton (Dotsenko and Mikhailenko, 2019), and to a lesser extent in the Daya Bay (South China), with a lower average concentration of 8.9 mg kg^{-1} d.w. (Qiu, 2015). From these figures, we can consider oyster spat to be fed, in the present study, with phytoplankton presenting a Cu load relevant from the environmental point of view.

Cu can be adsorbed on the microalgal cell wall through ion exchange and complexation. This is due to the negative charge of the cell wall related to the presence of different groups (e.g. carboxyl, amino and hydroxyl) on its surface, as has been reported for diatoms (Zhou et al., 2016). By preventing metal ions from penetrating the cell, complexation of metals to the cell wall is known to counteract metal internalisation and potential toxicity. Whereas Cu adsorption to cell surfaces is considered to occur in a few minutes and reach a pseudo-

equilibrium with Cu in solution, internalisation (i.e. absorption) of the metal across the membrane is much slower (Gonzalez-Dàvila,1995) and little is known about the site and mode of action of Cu in microalgae (Stauber and Davies, 2000). It is important to remember that Cu is an essential cofactor of photosynthetic and respiratory redox proteins that are necessary for electron transfer and oxygen cycling (Masmoudi et al., 2013). It is thus necessarily absorbed by phytoplankton.

4.2. Trophic transfer of Cu from *T. suecica* to *C. gigas* oyster spat

The hatchery spat used for the experiment presented a high initial Cu concentration (140 ± 14 mg.kg^{-1}) that was totally unexpected due to their origin. For comparison, Cu concentrations ranging from 30 to 140 mg.kg^{-1} dry weight were measured in farmed pacific oysters *C. gigas* from the Gulf of California coast (Jonathan et al., 2017) and from 27.6 to 163.8 mg.kg^{-1} dry weight in wild Pacific oysters collected along the Italian coast (Burioli et al., 2017). Although the Cu concentration can decrease by biodilution during spat growth, such a high concentration was surprising and we failed to identify the source of contamination. Neither the Cu content of the phytoplankton cultures used for spat production and feeding during the acclimation period (0.32 ± 0.17 and 18.27 ± 3.08 µg Cu.L^{-1} in the outdoor pond culture used at the hatchery and in the GreenSea concentrated culture, respectively), nor the Cu concentration in the seawater used at the hatchery could explain such contamination. The hatchery seawater had a mean Cu concentration of 343.5 ± 70.0 ng.L^{-1}, which is in the same concentration range reported in the Arcachon Basin (between 160 and 600 ng.L^{-1}) (unpublished results) and in the Gulf of Lion (around 200 ng.L^{-1}) (Chouvelon et al., 2019). These concentrations are similar to those measured in other European coastal waters, such as in protected (32–288 ng.L^{-1}) and unprotected (40–876 ng L^{-1}) coastal areas in Sardinia (Marras et al., 2020) and in the central zone of the Ligurian Sea (90–180 ng.L^{-1}) (Migon et al., 2020). Worldwide, Cu concentrations can even reach values close to µg.L^{-1} in more anthropized areas such as Peninsular Malaysia (Godon et al., 2018).

It is worth mentioning that during the exposure experiment, the contribution of dissolved Cu to spat contamination was negligible at least regarding the seawater concentration of dissolved Cu in the 'labile' form. The remaining culture medium brought into the tanks during the feeding of the oysters also did not contribute significantly to oyster spat contamination. The Cu concentrations measured with DGT in the assay tanks were not different from those measured in the control tanks, remaining at the environmental background level.

Due to this initial Cu contamination in spat, we recorded an evident depuration phase in the control group that could be directly related to spat growth. This depuration phase was more limited in the groups receiving Cu from their feed supply. The ability of bivalves to depurate metals has already been demonstrated (Anacleto et al., 2015). Han et al. (2015) showed that *C. gigas* depurated Cu rapidly (from 606 to 248 mg.kg^{-1} d.w. in 23 days in filtered seawater), with an estimated biological half-life of 25 days.

Regarding the DGT results, trophic exposure can reasonably be considered to be the predominant route of Cu exposure in this experiment. In the groups fed with contaminated phytoplankton, depuration was notably limited by concomitant trophic transfer of Cu. As indicated by the growth observed in all experimental groups, to a different extent depending on Cu contamination, contaminated phytoplankton were well consumed by spat. The trophic transfer of metals has already been studied in bivalves. Metian et al. (2019) discussed the composition of the oyster diet (phytoplankton, heterotrophic protists, large bacteria, fungi and detritus) and its possibility to retain particles > 4 mm. As demonstrated earlier by Dupuy et al. (1999), *C. gigas* retained and ingested 86% of flagellates (size between 4 and 72 µm) including *T. suecica,* the microalga used in the present study. Regarding Cu, Levy et al. (2007) showed that among the 11 algal species they studied, *Tetraselmis* sp. adsorbed the most Cu per cell (partition coefficient of $32 \times 10^{-10} \pm 1 \times 10^{-10}$ L.cell^{-1}) relative to the concentration of dissolved Cu in solution. It is worth mentioning that several field studies suggest the possibility for Cu to be transferred within the food web and especially in the first trophic levels. Chouvelon et al. (2019) showed that for Cu, Ni and Ag, the bioaccumulation

factor (BAF, the ratio between the element concentration in biological organisms and its concentration in seawater) continuously decreased from the smallest phytoplankton fraction (6–60 µm) up to two major small pelagic fish species. They proposed increased cell and organism size as the main driver decreasing direct adsorption and absorption in phytoplankton. By analysing 36 marine species from Chilean Patagonia and Antarctic Peninsula including the mollusk bivalve *Aulacomya atra*, Espejo et al. (2020) confirmed a possible biomagnification of Cu but only within the lower-trophic-level organisms, with its concentration decreasing from macroinvertebrates to fish or birds.

4.3. Cu slightly affected the physiology of *T. suecica* cultures

Cu exposure did not cause marked effects on the physiology of *T. suecica*. As mentioned previously, among trace metal elements, Cu is essential for many cellular functions. This is probably the main argument for its low toxicity, even though concentrations above the optimum level can become toxic (Baron et al., 1995). When some marine diatoms were shown to have a 72-h or 96-h half-maximal effective concentration (EC_{50}) close to 1,000 µg Cu.L^{-1} (Koutsaftis and Ayoma, 2006; Bao et al., 2008), some other phyla appeared much more sensitive to Cu considering its effect on algal growth rate. Levy et al. (2007) studied the toxicity of Cu in 11 algal species from different taxonomic groups including *Tetraselmis* sp. They first demonstrated a different effect of Cu on the algal growth rate depending on the species. They measured the 72-h half-maximal inhibitory concentration (IC_{50}) ranging from 0.6 µg Cu.L^{-1} for the small centric diatom *Minutocellus polymorphus* to 530 µg.L^{-1} for the tolerant chlorophyte *Dunaliella tertiolecta*. The microalga *Tetraselmis* sp. was found to be moderately sensitive, with a 72-h IC_{50} of 47 µg.L^{-1} and a lowest observed effect concentration (LOEC) of 22 µg.L^{-1}. Considering the exposure concentrations and duration of exposure in the present study, the lack of significant growth inhibition in Cu15-exposed *T. suecica* cultures is not surprising. At 50 µg.L^{-1}, a growth inhibition might have been expected. Stauber and Florence (1987) studied how Cu can disrupt algal growth. Once inside the cytoplasm, they showed that Cu can decrease the ratio of reduced to oxidised glutathione, leading to

impairment of spindle formation and cell division. Cu could also be involved in electron transport inhibition, namely by damaging acceptor and donor sides of the photosystem II (Patsikka et al., 1998), which would decrease the efficiency of photosynthesis (El Berdey et al., 2000).

It is worth mentioning that differences in sensitivity can also be observed within the same genus. Ismail et al. (2002) found that *Tetraselmis* sp. was three times less sensitive than *Tetraselmis tetrahele*, with 96-h IC_{50} values of 370 and 130 $\mu g.L^{-1}$ respectively. Interspecies differences in Cu sensitivity were not related to cell wall type, cell size, taxonomic class and equilibrium partitioning of Cu to the cell, but rather to Cu uptake rates and intracellular detoxification processes (Levy et al., 2007). The latter include direct (active efflux mechanisms) and indirect (release of exudates to reduce bioavailability, reduced membrane permeability) exclusion and internal sequestration (Megharaj et al., 2003). Once Cu is internalised, cysteine-rich phytochelatins can bind excess Cu, rendering it less toxic through subcellular partitioning of metals to inactive sites. Kong and Price (2020) used proteomics to identify Cu-regulated proteins in the diatom *Thalassiosira oceanica* when grown in Cu-limited batch cultures. They focused on the main cuproproteins that contain most of the metabolically active Cu such as plastocyanin (an electron carrier involved in photosynthesis), 8,9 cytochrome *c* oxidase (terminal enzyme in the respiratory electron transport chain) and the multicopper oxidase (involved in Fe assimilation pathway). They identified 73 differentially expressed proteins in Cu-limited cells: 28 were upregulated (involved in genetic information processing, lipid metabolism and stress response) and 45 were downregulated (involved in photosynthesis, carbon, nitrogen or sulphur metabolism), including cuproproteins such as plastocyanin. Although Kong and Price (2020) investigated the effect of a Cu depletion rather than Cu contamination as done in the present study, their results provide information on the possible physiological impact of Cu and the nature of proteins whose synthesis may be modulated if the Cu concentration changes. In the present study, despite a strong variability between days and cultures, Cu-exposed cultures could be distinguished from control by

presenting lower metabolic activity and lipid content as well as with higher relative intracellular ROS content, as evidenced by HE and DCFH fluorescence values obtained by flow cytometry. These results are congruent with other studies on Cu effects on microalgae. For example, after exposure of the marine microalga *Pavlova viridis* to 0–3 mg.L^{-1} Cu^{2+} for about 2 weeks, Li et al. (2006) evidenced a significant increase in CAT activity to 50 µg.L^{-1}, while the other antioxidant enzymes were only stimulated at the highest Cu concentrations. CAT can scavenge H_2O_2, which is one of the ROS species evidenced by the fluorochrome DCFH-DA used in our measurements. Sabatini et al. (2009) also demonstrated an increase in the antioxidant defences of the freshwater microalga *Scenedesmus vacuolatus*, probably corresponding to oxidative stress, after exposure to copper chloride concentrations in the mg.L^{-1} range. These high concentrations may look surprising, but the culture medium used by the authors contained EDTA, which was lacking in the present study. Hamed et al. (2017) showed an increase in the H_2O_2 content of *Chlorella sorokiniana* and *Scenedesmus acuminatus* by 1.9 and 1.7 fold, respectively, as well as approximately 2-fold increase in TBARS after 1-week exposure to 1.6–3.2 mg.L^{-1} of Cu. Finally, Jamers et al. (2013) observed an increase in the relative intracellular ROS content of the freshwater microalga *Chlamydomonas reinhardtii* after 48-h exposure to 0.27 µg.L^{-1} of free Cu^{2+} ions, by using the DHR123 fluorochrome measured by flow cytometry.

Cu exposure of *T. suecica* cultures also induced effects on NL, as indicated by flow cytometry analyses. In detail, lipid composition analyses revealed impacts on FA, which are synthesised as building blocks for the formation of various types of lipids. FA are either saturated or unsaturated, and the latter may vary in number and position of double bonds on the carbon chain backbone. In nine marine species, including *T. suecica*, Ohse et al. (2015) also reported synthesis of PUFA and MUFA with some differences among species in the ratio between these two types of FA. In the present study, there was an increase in all lipid classes between day 7 and 8 in both control and Cu-exposed cultures of *T. suecica*, indicating non-limiting conditions for algal growth and metabolic activities. Lipid profiles

changed markedly within a 24-h period, as illustrated by the modifications we observed concerning the correlations between the different lipid classes. Such changes with the growth phase were already reported in *Tisochrysis lutea* (Da Costa et al., 2017). Lipid metabolism in microalgae has been shown to be affected by several environmental factors. The effect of nitrogen limitation has been widely studied due to the interest of using algae to produce biodiesel. By working on 11 algae species including *T. suecica*, Griffith et al. (2011) showed that nitrogen-limited conditions increase the lipid content and the proportion of SFA and MUFA while decreasing the proportion of PUFA. The effects of salinity and pH were also reported in this species with an increased production of MUFA and one PUFA under salinity and pH stresses (Almutairi and Toulibah, 2017). Chemical pollution is also a factor influencing lipid metabolism in algae. When *Chlorella vulgaris* was grown in nitrogen-replete conditions, the addition of Cd in the culture medium (two tested concentrations of 2.0 x 10^8 and 1.0 x 10^7 M) increased all lipid classes, and there were correlations between Cd concentrations and 18:2(n-6) and 18:1(n-7) (positive correlations) and phospholipids, aliphatic hydrocarbon, free sterols, 14:0 and 18:0 (negative correlations) (Chia et al., 2013). Kong and Price (2020) showed that a change in the Cu supply affected FA metabolism in the diatom *T. oceanica*. In Cu-limited cultures (1 nM Cu), long-chain fatty acid CoA synthetase and acyl-CoA dehydrogenase were strongly upregulated probably due to increased cycling of lipids and FA in response to oxidative damage to cell membrane. Focusing on metabolomics, Gauthier et al. (2020) also reviewed the reported effects of different pesticides and metals on the lipid metabolism of microalgae. A wide range of metabolites from various chemical structures (amino acids, FA, carbohydrates, quaternary amines, organic acids and terpenoids) exhibited significant changes in abundance due to chemical stress. Among FA, they underlined that SFA and MUFA are the main source of stored energy in microalgae and that an increase in MUFA could be explained by photosynthesis inhibition. In the present study, Cu seemed to increase the content of SFA 15:0 and 20:0 and MUFA 16:1n-5, changes that could be associated with a possible decrease in photosynthesis and energy cost. Such an increase in the SFA content (e.g. palmitic acid 16:0 and stearic acid 18:0) was also

observed in *Thalassiosira weissflogii* following exposure to Cu (0.2–0.8 mg. L^{-1}), with an opposite trend for unsaturated FA (Filimonova et al., 2016). This change in the FA profile was assumed as a means for algae to counterbalance the damage induced by ROS. It is consistent with the higher ROS production (based on flow cytometry) observed in the present study in Cu-exposed phytoplankton cultures.

It is noteworthy that Cu exposure seemed to decrease the content of PUFA 22:6n-3, also known as DHA. Alongside eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6), DHA is one of the FA that plays a key role in the maintenance of physiological functions of many organisms, providing energy and being a major constituent in the cellular wall.

4.4. In spat, a decrease in the condition index could be related to changes in the fatty acid contents of the food supply

The decrease in glycogen content in all groups, including the control, could indicate that the amount of feed provided during the experiment was not sufficient despite a significant growth of total individual weight over time (15% increase, data not shown). Following Cu exposure, the Ci decreased compared with the control animals. This negative impact on the general health condition of oyster spat could be related to the effect of Cu exposure on the FA content of the phytoplankton cultures used for feeding. In fact, cultures exposed to Cu presented a tendency to lower DHA content. As mentioned previously, DHA is an essential FA and plays a key role in larval development, growth and reproduction in bivalve molluscs. Costa Gonzalez et al. (2017) accurately quantified the FA contained in algae used as a feeding source for *C. gigas* juveniles to measure their assimilation in the oyster. Their results evidenced that oyster larvae efficiently absorbed these essential FA from algae. They also suggested the possibility for oyster larvae to synthesise low amounts of ARA and EPA from precursor FA when present in an insufficient quantity in the food supply. On the contrary, they confirmed the inability of oysters to synthesize DHA from shorter-chain precursors. As a

consequence, DHA must be considered as an essential dietary nutrient for growth, reproduction and neural development of bivalves. Hendricks et al. (2003) studied the effect of PUFA on the reproduction of *Macoma balthica*. In adults fed with a diet enriched in PUFA, spawning percentages were higher and females produced markedly more eggs. In the juvenile ark shell *Tegillarca granosa*, the abundances of AA, EPA, and DHA affected growth and were directly dependent on the unialgal diet selected; animals fed with *Chaetoceros calcitrans*, *Isochrysis galbana* or *Pavlova viridis* presented higher growth (Xu et al., 2012).

It is noteworthy that some mollusc species operate alternative and unusual pathways of PUFA biosynthesis (Monroig and Kabeya, 2018). In the present study, such classes of genes were not investigated through gene expression analysis. As indicated by oyster Ci, there was significant growth of spat in the control condition despite a marked decrease in glycogen content. In a study on *C. gigas* adult oysters, Parnarun et al. (2003) showed an increase in the glycogen and lipid content of oysters fed with *I. galbana* (clone T-iso, recently renamed *T. lutea*) or *S. costatum* (recently renamed *S. marinoi*). Their results indicated a good availability and assimilation of microalgae, which was obviously not the case in the present study. Even though all the *T. suecica* cells were consumed every 24 h in the tanks, we can indeed assume *T. suecica* is not the ideal monospecific diet for energy storage. Brown et al. (1998) tested several diets on *C. gigas* spat (about 1,800 µm in width and 2.4 mg d.w.) over 21 days. The best growth of spat, expressed as an increase in organic weight, was obtained by using a mixed diet of five microalgae, including *Tetraselmis* sp. However, oyster spat fed a *Tetraselmis* sp. monospecific diet, as in our study, resulted in a much lower growth (51% increase vs 35%–111% increase induced with the other monospecific diets tested). *Tetraselmis* sp. was also the microalga with the lowest lipid content (16% ± 2% vs 16%–26% d.w. in the five species tested). The authors explained that *Tetraselmis sp*. does not generally contain DHA, which is essential for mollusc nutrition (Langdon and Waldock, 1981; Volkman et al., 1989, 1993; Brown et al., 1998). In another study on the mangrove oyster *Crassostrea corteziensis,* juveniles (5 weeks after settlement, 0.36 g and 4.6 mm) were fed a

monospecific diet or a binary microalgal diets, including *T. suecica*, for 22 days (Rivero-Rodríguez et al. 2007). Of the five microalgal species tested, *T. suecica* had the lowest nutritional value and yielded the lowest oyster growth rate. It also showed a notably lower amount of highly unsaturated FA than the other species tested. In particular, there was a lack of DHA alongside low levels of ARA and EPA, findings the authors used to explain its low nutritional value. The decrease in glycogen content in this study is thus not surprising. Our choice to use *T. suecica* as a monospecific diet for oyster spat was mainly driven by experimental constraints. Even though the oysters were not able to store energy, they were still growing. They presented an apparent state of good physiological conditions, all the more they were not challenged by other stressors during the experiment.

It is noteworthy that DHA was detected in both control and Cu-exposed *T. suecica* cells, demonstrating this species has a low but existing capability to synthesize this essential FA.

4.5. Gene expression also suggests a link between the decrease in spat Ci and the observed energy demand

In this study, numerous genes were differentially expressed in the Cu-exposed compared with control individuals, mainly after 2 and 3 weeks of exposure (days 14 and 21). This time-dependent response could be linked to the absorption of Cu in spat during the time course of the experiment. Indeed, at day 14 and 21, the Cu concentration was significantly higher in animals fed with contaminated phytoplankton than in those fed with the control phytoplankton. Such differences were also in agreement with the induction of copper transporter 2, which plays a role in the entry of this metal into the cells. Various reports have evidenced that oysters accumulate and tolerate high levels of trace metals such as Cu, Zn, Cd or Hg (Wang et al., 2018). This capacity has been linked to their ability to mobilise detoxification mechanisms. Trace metal detoxification could be performed via intracellular chelation, transport to mitochondria, isolation into lysosomes and excretion out of the cell by active efflux pumps (i.e. ABC transporters). Interestingly, we observed overexpression of

metallothionein (MT) genes (*mt1* and *mt2*) and *vdac2,* which is involved in transport to the mitochondria. MT are low-molecular-weight, cysteine-rich proteins involved in homeostasis of essential metals and scavenging and detoxification of toxic metals (Bourdineaud et al., 2006; Capdevila et al., 2012). Their overexpression in oyster spat after 14 and 21 days of exposure to Cu suggests the implementation of detoxification mechanisms. Once linked to MT, metals could be transported to the lysosomes and then excreted as previously reported in the mussel *Mytilus edulis* (Marigomez et al., 2002) or the green oyster *Crassostrea sikamea* (Wang and Wang, 2014).

Cu is an essential metal for most living organisms, but it can become toxic above a certain concentration. Previous studies have highlighted that once in the cell, Cu could exert toxic effects mainly by inducing an oxidative stress and leading to DNA damage, lipid peroxidation and apoptosis (Fitzpatrick et al., 2008; Gamain et al., 2017). In this study, the expression of genes involved in stress response (*hsp70*, *cox2*), DNA repair (*gadd45*, *xrcc4*) and apoptosis (*casp9*) was elevated, suggesting toxic effects of Cu in juvenile oysters after 14 and 21 days of exposure to Cu. Because antioxidant genes (*sod*, *cat*, *gpx*) were not differentially expressed, the overexpression of *mt1* and *mt2* could have a role in counteracting oxidative stress. Indeed, besides their natural role in homeostasis and scavenging of metals, numerous other functions have been described for MT. For example, MT can be involved in the oxidative stress defence (Ruttkay-Nedecky et al., 2013). Indeed, we speculate that at the end of the experiment, the increased expression of MT genes could have been sufficient to chelate excess Cu, thus reducing oxidative stress.

Gene expression also indicated an increase in energy demand. Glycogen phosphorylase (glycogen catabolism) was overexpressed in the spat exposed to Cu15 and Cu50 conditions at day 14, and *ptl2*, a pancreatic lipase, was overexpressed in the spat exposed to the Cu50 condition at day 21. Pancreatic lipases are key enzymes involved in lipid digestion, storage and mobilisation of reserves during fasting or heightened metabolic demand (Rivera-Perez 2015). The overexpression of genes involved in mitochondrial and energy metabolism could

indicate an increased cell energy demand that is consistent with the overexpression of ATP synthesis and glycogen catabolism genes. In the oyster *Crassostrea virginica*, one month exposure to 50 µg.L^{-1} of Cd led to a significant decrease in tissue-stored glycogen as a result of increased free glucose levels and high glycolytic enzyme activities (Ivanina et al., 2011). In the same way, Liu and Wang (2016) demonstrated a clear relationship between bioaccumulation of Cu, Zn and Ni and a decrease in the glycogen content in the euryhaline oyster *Crassostrea hongkongensis* harvested in the Pearl River estuary, where they were subjected to polymetallic contamination. Finally, exposure to Cd led to a dramatic decrease in glycogen stores (between 61% and 72%) in the freshwater bivalve *Lamellidens marginalis* (Das and Jana, 2003). Regarding Cu, an increase in energy metabolism has been reported in the marine clam *Mesodesma mactroides* to allow the maintenance of cellular ATP production (Giacomin et al., 2014). In this study, there was a concomitant time-dependent decrease in the glycogen content regardless of the Cu exposure concentration and a lower Ci for Cu-exposed individuals at the end of the experiment. Given these results, we hypothesise that exposed spat used the extra energy demand (overexpression of genes involved in energy metabolism, ATP synthesis and glycogen catabolism) to cope with the toxic Cu effects. This change in energy allocation has been at the expense of the growth of the individuals. A low Ci has been previously reported for many oyster species harvested in metal-polluted areas in Australia (Avery et al., 1996), New Zealand (Pridmore et al., 1990) and France (Séguin et al. 2016).

5. Conclusion

In the present paper, a complex experimental approach was employed to study the trophic transfer of Cu between a microalga, *T. suecica*, and spat of a marine bivalve, *C. gigas*. A first remarkable result is that our experimental Cu-exposure of phytoplankton cells made it

possible to reach Cu levels in cells comparable to those found in marine environments. This trophic exposure of oysters can thus be considered as environmentally relevant. This is a major point when studying the trophic transfer of contaminants in controlled conditions, which remains a big but necessary challenge to better assess the risk associated with secondary poisoning.

Microalgae exposure to Cu highlighted impacts on fatty acids, confirming that copper contamination is a factor influencing lipid metabolism in microalgae. Indeed, when Cu exposure tended to increase the content of SFA 15:0 and 20:0, and MUFA 16:1n-5, which could be associated with a possible decrease in photosynthesis and energetic cost, a decrease in the content of the essential DHA was also noted. This change is suspected to have negatively impacted the general health condition of oyster spat during our study, which could have been further confirmed with lipid analyses of spat tissues to check for their lipid profiles and reserve status, for instance. FA composition is commonly used as a trophic marker and such an impact of chemical contamination demonstrated its potential to affect trophic ecology and thus the diversity and functioning of ecosystems.

The design of a long-term experiment would allow us to better investigate the impacts of a chemical contamination of oyster feed on their development and reproduction. Finally, in the context of global change, it seems necessary to investigate more deeply the influence of some environmental variables such as temperature and pH on the direct and indirect effects of chemical contamination by trophic transfer on the health of marine organisms.

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7. Author contributions

F. Akcha: Funding acquisition, project administration, conceptualisation, sampling and genotoxicity analysis, main contributor to writing the paper.

N. Coquillé: Conceptualisation, sampling, phytoplankton cultures, flow cytometry and gene expression analysis in phytoplankton.

R. Sussarellu: Sampling, supervision of gene expression analysis in phytoplankton and spat.

J. Rouxel: Sampling, catalase activity, glycogen content and gene expression analysis in spat.

T. Chouvelon: Sampling, supervision of copper analysis in culture medium and phytoplankton.

P. Gonzalez: Sampling, supervision of gene expression analysis in phytoplankton and spat.

A. Legeay: Sampling, supervision of copper analysis in spat, analysis of thiobarbituric acid reactive substances in spat.

S. Bruzac and T. Sireau: Sampling, copper analysis in culture medium and phytoplankton pellets.

J-L. Gonzalez: Diffusive gradient in thin film data analysis.

P-Y. Gourves: Sampling, analysis of copper in spat.

Y. Godfrin: Sampling, gene expression analysis in phytoplankton.

V. Buchet: Sampling, logistics at Ifremer Bouin.

S. Stachowski-Haberkorn: Conceptualisation, sampling, phytoplankton cultures and flow cytometry analysis, main contributor to writing the paper.

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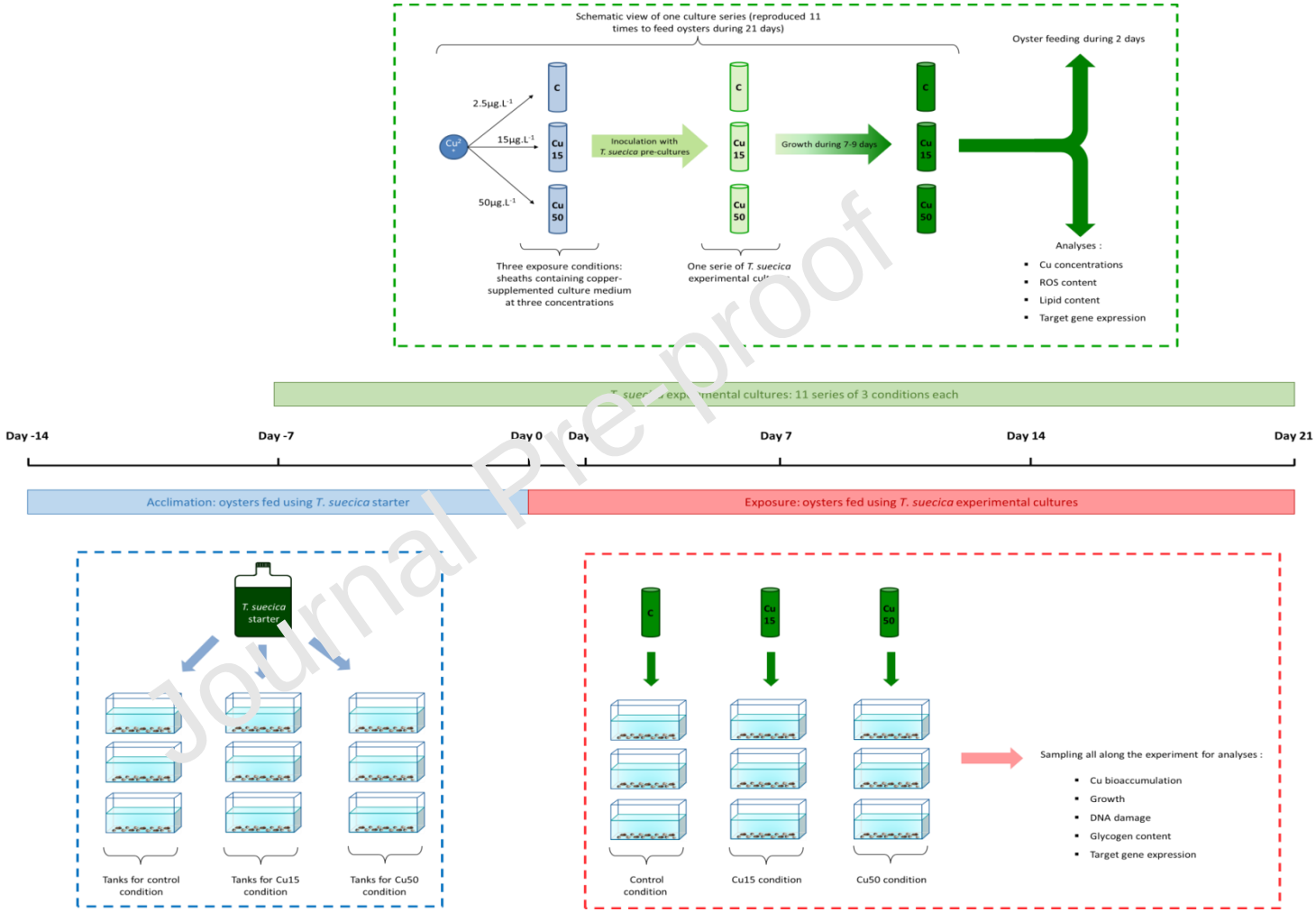


Figure 1: Schematic view of the time-course of experiment, from day -14 to day 21, representing the two components. The first one, concerning *T. suecica* experimental feeding cultures (in green), represents the duration covered by the growth and exposure of the 11 consecutive series, each composed of three treatments (C, Cu15 and Cu50). The second one, concerning oysters, includes acclimation phase (in blue) and exposure phase (in red).

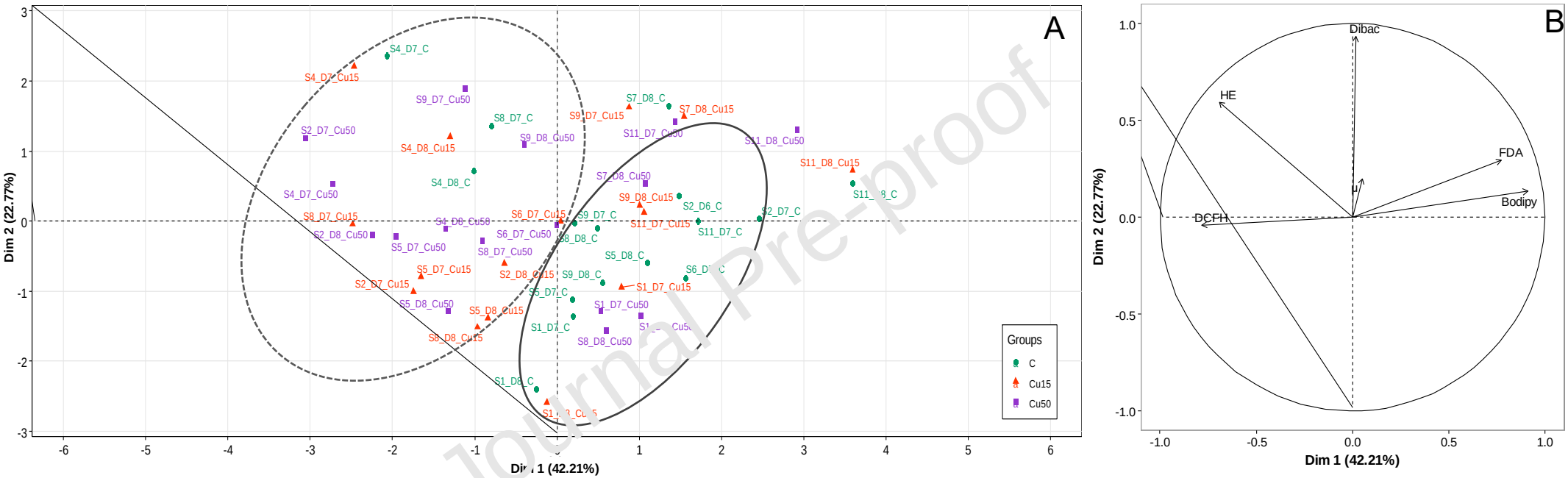


Figure 2: Principal component analysis (PCA) illustrating the relationships between biological parameters of T. suecica cultures measured by flow cytometry (μ: growth rate; Bodipy: relative intracellular lipid content; HE and DCFH: presence of intracellular reactive oxygen species, FDA: metabolic activity, Dibac: cytoplasmic membrane potential) in control (C), Cu15 and Cu50 (Cu at 15 µg.L-1 and 50 µg.L−1, respectively) conditions for each series (S) of cultures used for oyster feeding after 7 and 8 days of growth (D7 and D8, respectively). A: corresponds to the projection of individuals; ellipses were added afterwards in order to illustrate the overall trends observed in the repartition of samples per condition (full line for C, hatched line for Cu15 and Cu50). B: corresponds to the factor map of variables.

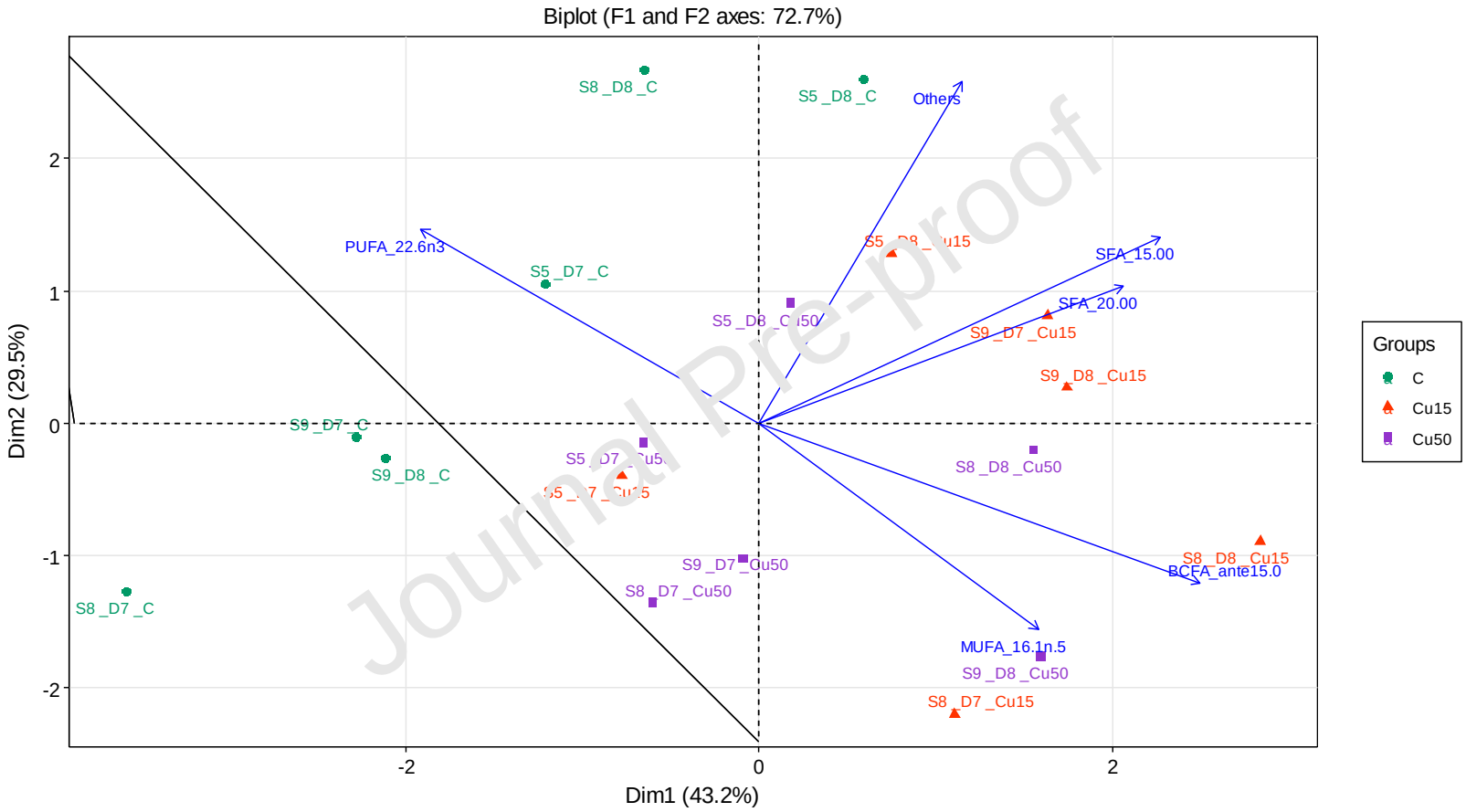


Figure 3: Observations and variable biplot from PCA based on data collected for each culture for cell content in PUFA 22:6n-3, SFA 15:0 and 20:0, BCFA ante 15:0, MUFA 16:1n-5 and sum of other quantified fatty acids. Data are presented for the three conditions, control (C), Cu at

15 µg.L^{-1} and 50 µg.L^{-1} (Cu15 and Cu50 respectively), series of cultures (S5, S8, S9) used for oyster feeding after 7 and 8 days of growth (D7 and D8, respectively).

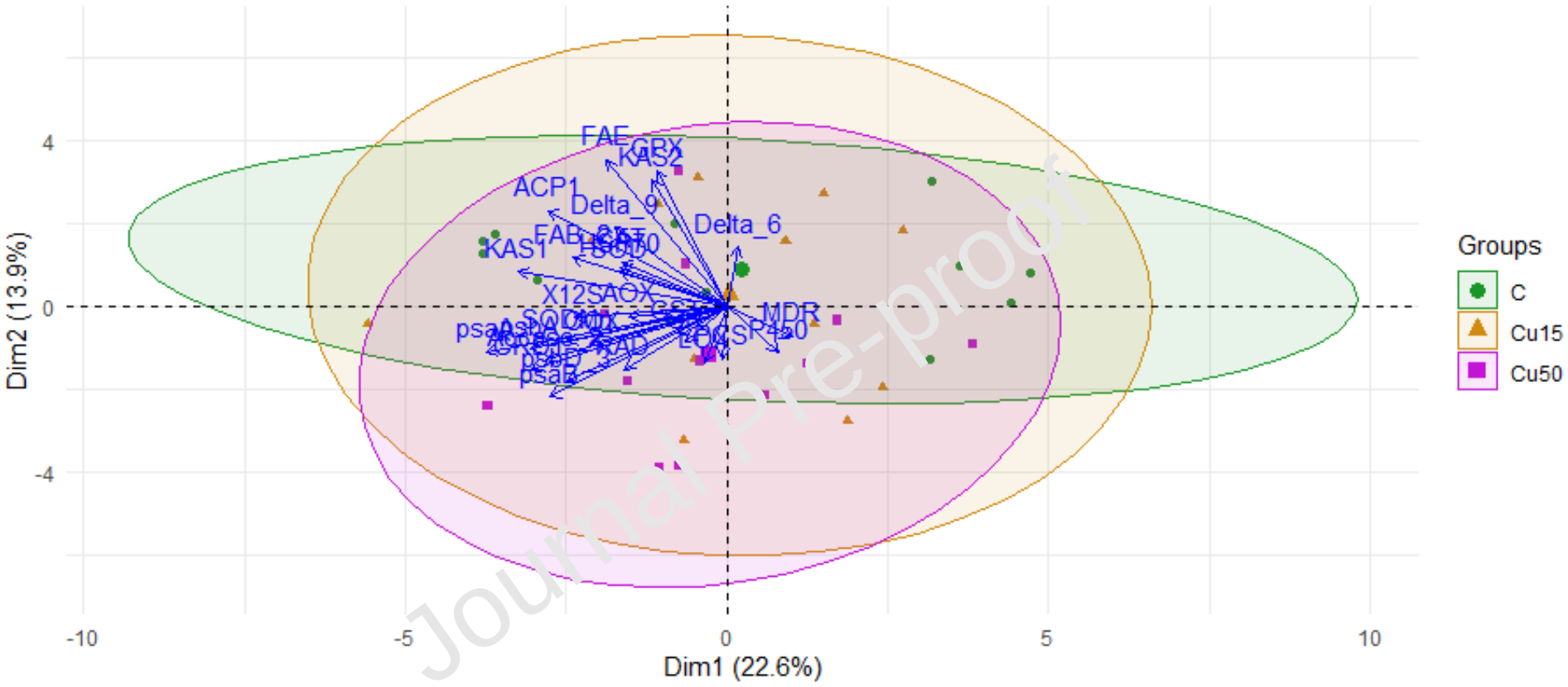


Figure 4: PCA analysis for target gene expression in *T. suecica* exposed to copper.

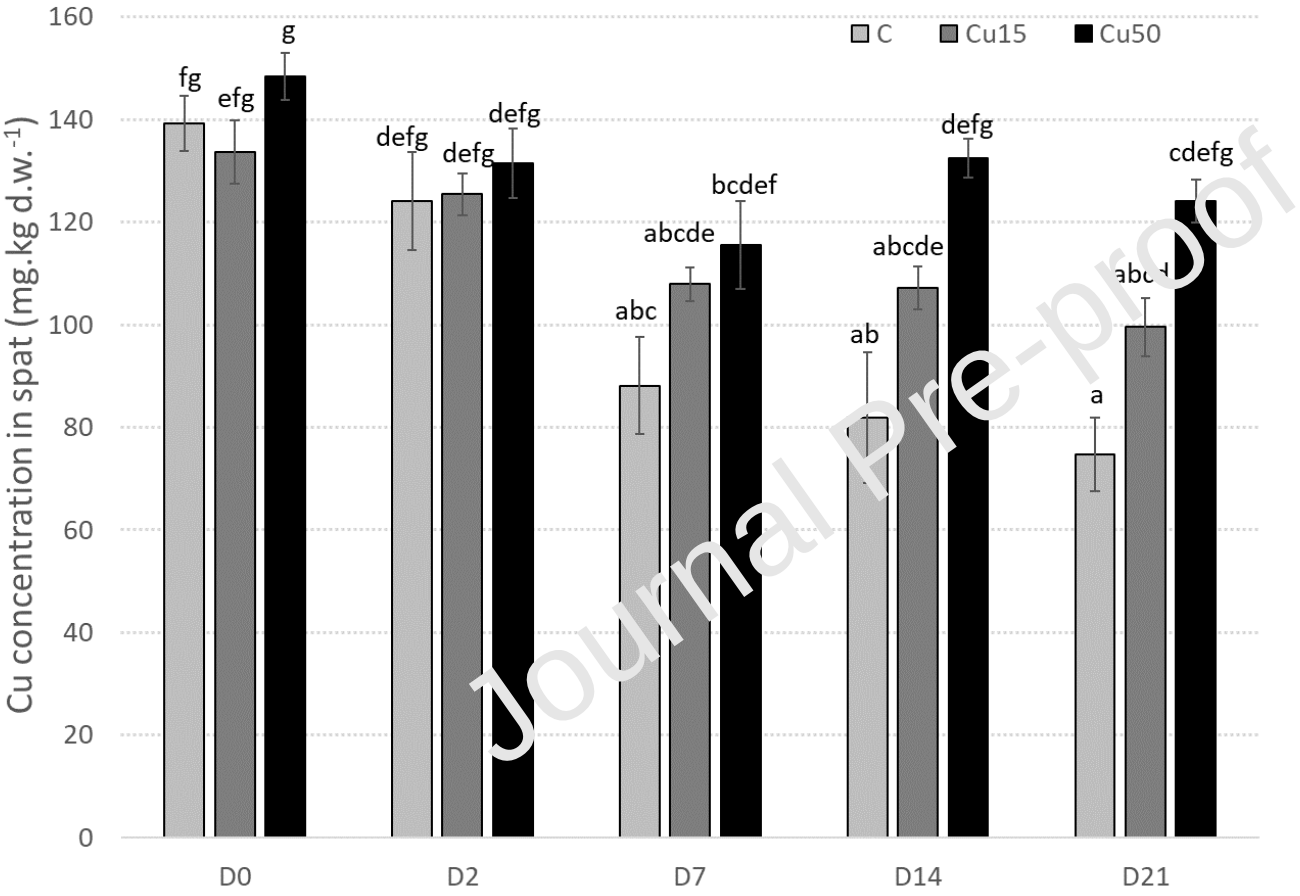


Figure 5: Copper concentrations (mg.kg^{-1} dry weight) measured in spat tissues for control, Cu15 and Cu50 conditions at each sampling time (mean ± SE, N=6). Data that do not share the same letter are significantly different ($p<0.05$).

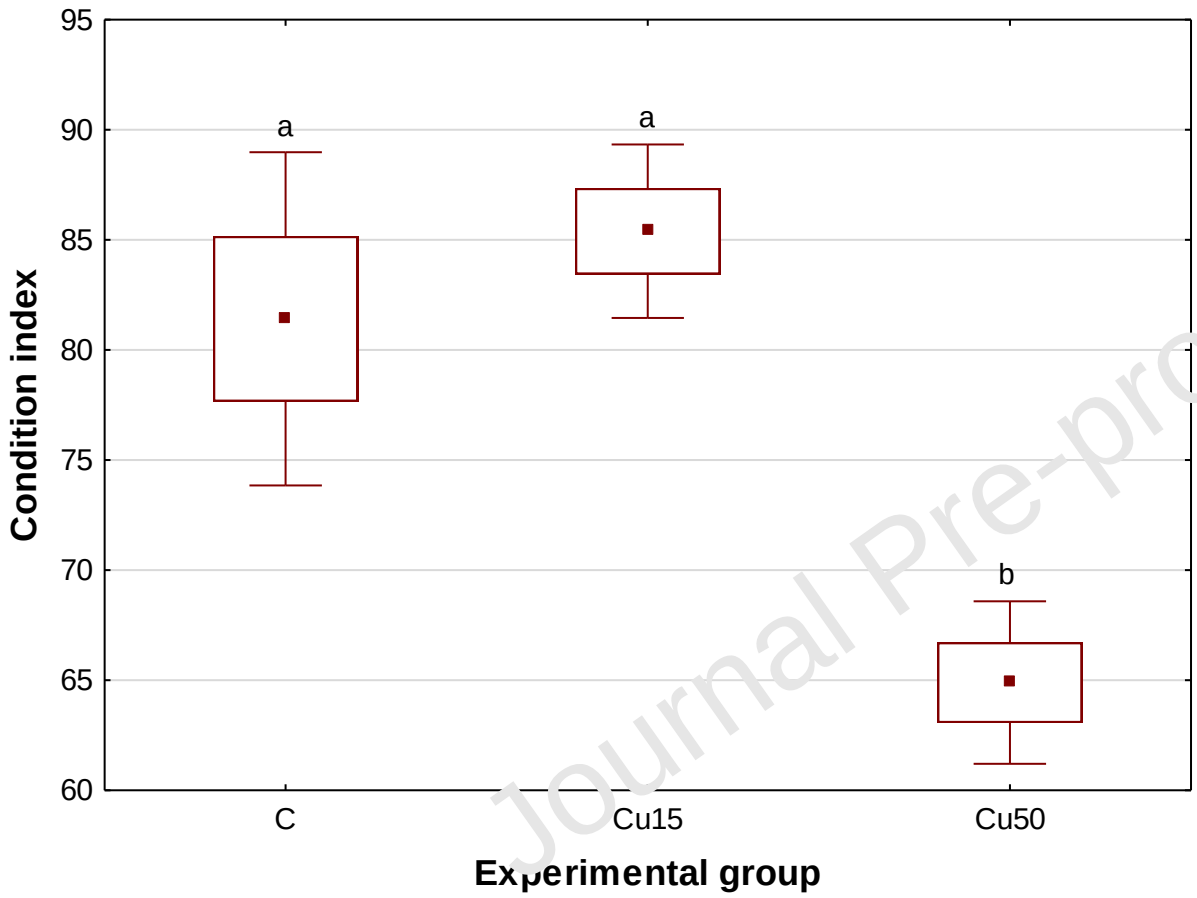


Figure 6: Condition index values obtained at the end of the 21 day-exposure in juvenile oysters fed with control and copper-exposed phytoplankton cultures. Means are represented with their standard error and 0.95 confidence interval. Data that do not share the same letter are significantly different ($p<0.05$).

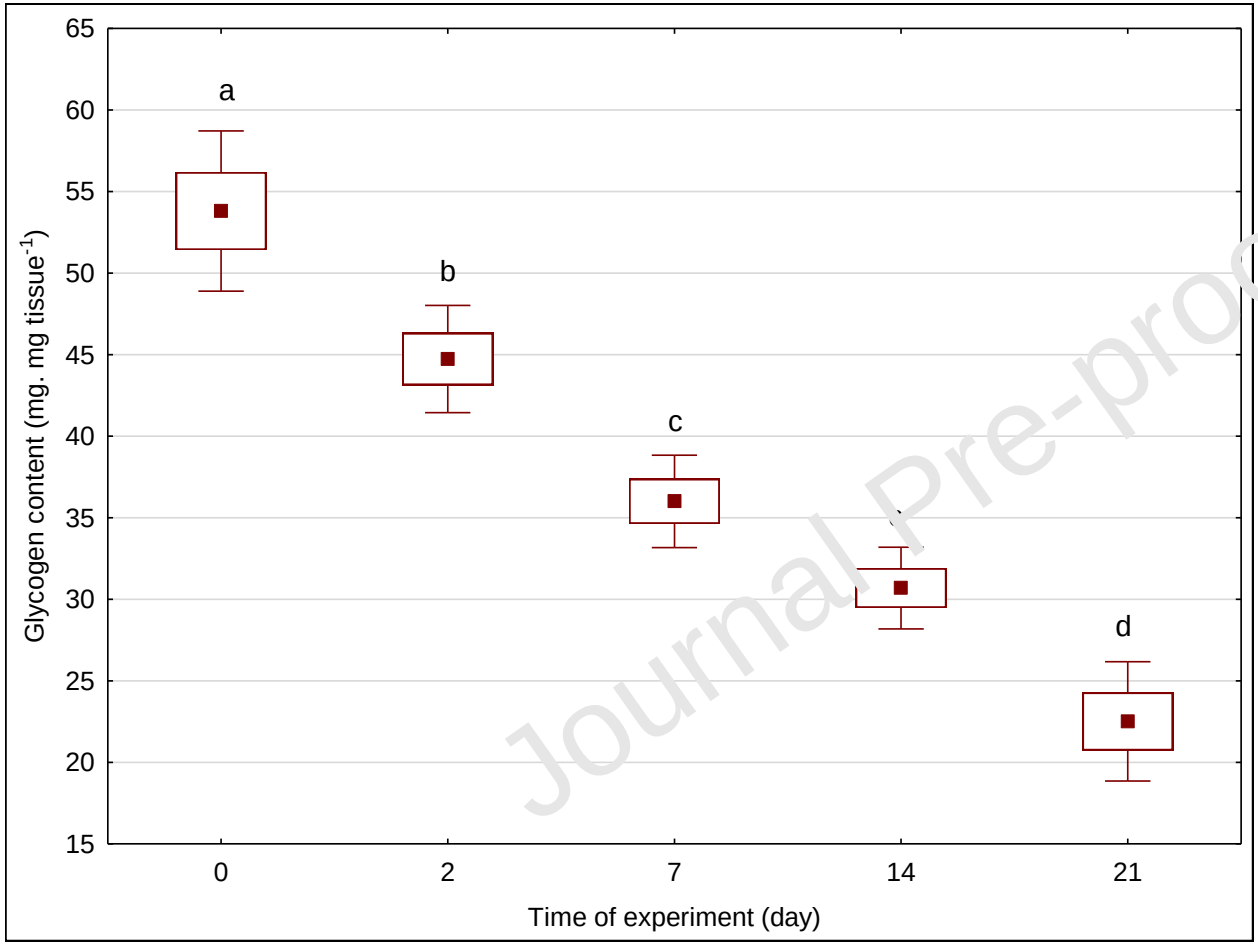


Figure 7: Mean effect of experiment time on the glycogen content of oyster spat. Means are represented with their standard error and 0.95 confidence interval. Data that do not share the same letter are significantly different ($p<0.05$).

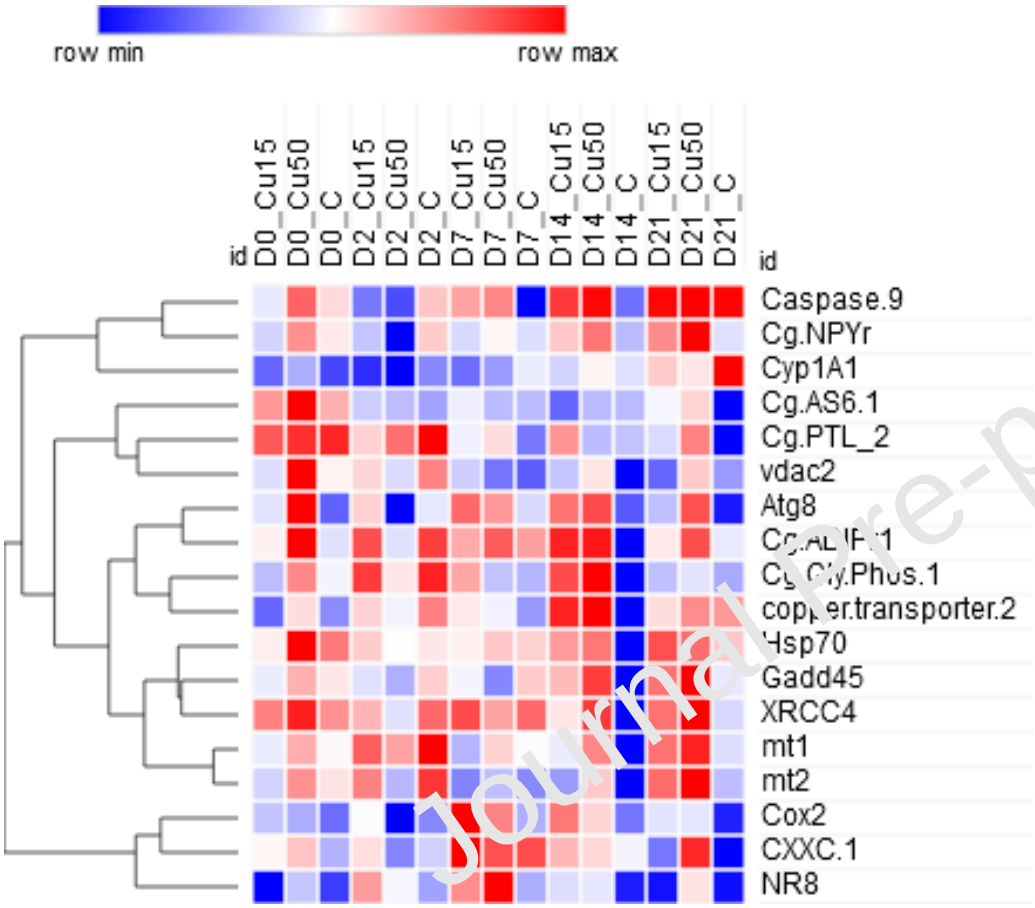


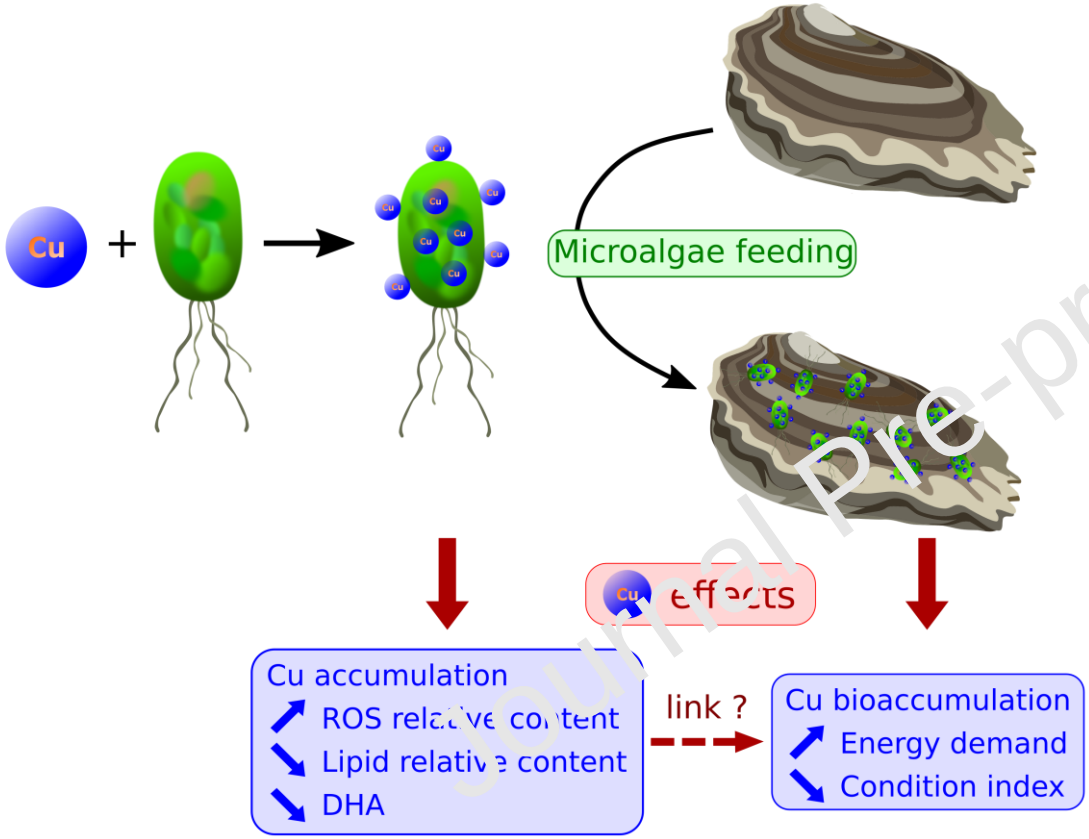
Figure 8: Heat map for the 18 genes showing time / condition significant effects ($p<0.05$) according to the nested ANOVA. Each square represents the mean expression of biological replicates for a given sampling time, N=9. The graph was obtained with Morpheus (https://software.broadinstitute.org/morpheus) using 1-correlation (Pearson) distance and linkage average.

Table 1: Statistical analysis performed for chemical and biological data collected on both phytoplankton and oyster spat, $p < 0.05$ considered statistically significant.

Species	Parameters	Statistical analyses	Software/packages
T. suecica	Cu concentrations	Between day 0 and day 7–8 was assessed for each condition with Student's t-test after having checked the homoscedasticity with Fisher's F-test	R 3.6.3. (R Core Team, 2020)
	Growth rates and physiological parameters	PCA	R 3.6.3. (R Core Team, 2020) +R packages FactoMineR (Husson et al., 2020), factoextra (Kassambara and Mundt, 2017) and ggplot2 (Wickham H., 2021)
	Lipid content	PCA	
	Target gene expression	PCA	
C. gigas	Cu concentrations	A Box–Cox transformation (Peltier et al., 1998) was performed to test the normality (with Shapiro's test) and homogeneity (with Cochran's test) of the data. Subsequently, a two-factor analysis of variance (ANOVA) followed by Tukey's test was performed.	Statistica Soft.14.0
	CI index	At the end of the 21-day exposure experiment, the Ci values calculated for individuals from each experimental group were compared using a nested-design ANOVA (replicates being nested in the experimental group) followed by Tukey's test. Normality and homoscedasticity were previously checked by normal p-Plot and Hartley 's test.	Statistica Soft.14.0
	Glycogen content; CAT activity; Lipid peroxidation	Normality and homoscedasticity were previously checked by normal p-Plot and Hartley 's test. Each ANOVA was followed by a Tukey's test.	Statistica Soft.14.0
	DNA damage	Raw comet data were square-root transformed to fit a normal distribution and variance homogeneity was checked by Hartley's test. An ANOVA was realized.	Statistica Soft.14.0
	Target gene expression	Normality and homoscedasticity were previously	Statistica Soft. 14.0 + Morpheus

		checked by normal p-Plot and Hartley ‘s test. A nested ANOVA was realized. A heat map was proposed to visualize the results.	(https://software.broadinstitute.org/morpheus)

Graphical Abstract



Highlights

- Contaminated *T. suecica* cultures reached an environmentally relevant load of Cu
- Cu impacted the ROS content in algae, alongside their fatty acid profile
- Trophic transfer of Cu led to spat contamination close to what occurs in the field
- Spat fed with Cu-exposed algal cultures had a lower condition index
- Insufficient supply of PUFA 22:6n-3 and elevated energy demand may explain this effect